THE VITAMINS

VOLUME I

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THE VITAMINS

Chemistry, Physiology, Pathology

VOLUME I

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THE VITAMINS

Chemistry, Physiology, Pathology

VOLUME I

EDITED
BY

W. H. Sebrell, Jr.
Director, National Institutes of Health
Bethesda, Maryland

Robert S. Harris
Department of Food Technology
Massachusetts Institute of Technology
Cambridge, Massachusetts

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CONTRIBUTORS TO VOLUME I

Frank H. Bethell (491–506, 522–523)*
The Thomas Henry Simpson Memorial Institute for Medical Research
University of Michigan
Ann Arbor, Michigan

Karl A. Folkers (397–417, 443–448)
Organic and Biochemical Research Division
Merck & Co., Inc.
Rahway, New Jersey

Paul György (527–593, 595–618)
Department of Pediatrics
Hospital of the University of Pennsylvania
Philadelphia, Pennsylvania

Robert S. Harris (3, 179–180, 396, 526)
Department of Food Technology
Massachusetts Institute of Technology
Cambridge, Massachusetts

H. H. Inhoffen (87–106)
Organisch-Chemisches Institut der Technischen Hochschule
Braunschweig, Germany

Thomas H. Jukes (421–443, 448–491, 507–522)
Department of Nutrition and Physiology Research
Lederle Laboratories
Pearl River, New York

L. W. Mapson (211–242)
Low Temperature Research Station
Department of Scientific and Industrial Research
Cambridge, England

Karl E. Mason (137–163, 171–175)
Department of Anatomy
School of Medicine and Dentistry
University of Rochester
Rochester, New York

Fred H. Mattson (163–170)
Chemical Division
Miami Valley Laboratories
Procter and Gamble Co.
Cincinnati, Ohio

Nicholas A. Milas (4–58)
Department of Chemistry
Massachusetts Institute of Technology
Cambridge, Massachusetts

Mamie Olliver (242–268)
Chivers and Sons, Ltd.
Cambridge, England

H. Pommer (87–106)
Organisch-Chemisches Institut der Technischen Hochschule
Braunschweig, Germany

Mary Elizabeth Reid (269–347, 380–388, 391–393)
National Institutes of Health
Bethesda, Maryland

Fred Smith (180–211)
Division of Agricultural Biochemistry
University of Minnesota
St. Paul, Minnesota

Esmond E. Snell (593–595)
Department of Chemistry
University of Texas
Austin, Texas

Richard W. Vilter (348–380, 388–391)
Cincinnati General Hospital
Cincinnati, Ohio

George Wald (59–87)
Biological Laboratories
Harvard University
Cambridge, Massachusetts

William L. Williams (421–443, 448–491, 507–522)
Lederle Laboratories
Pearl River, New York

S. Burt Wolbach (106–137)
Division of Nutritional Research
The Children's Hospital
Boston, Massachusetts

Donald E. Wolf (397–417, 443–448)
Merck & Co., Inc.
Rahway, New Jersey

H. M. Wuest (417–421)
137 Summit Avenue
Montclair, New Jersey

* Numbers in parentheses indicate the pages on which each author's contribution or contributions may be found.

PREFACE

The story of vitamins resembles, in many ways, the story of the Tower of Babel. What once seemed simple has become confounded; what once could be mastered by a few is now only partly understood by the many. The very large number of publications dealing with the many aspects of vitamin research confronts the investigator and practitioner like a strange and newly discovered land, each area with its own special interests.

In this book the editors have attempted to provide, as it were, a guide service to these new and complicated areas. Just as guides are chosen for their special knowledge of each specific region, so each contributor to this work has been chosen for his competence in a specific field. For this reason no contributor has discussed one vitamin entirely, and some have discussed the same aspect of knowledge regarding several vitamins. This method will force the reader to change guides as he progresses through each chapter, but it will assure a higher level of competence than if one guide were to attempt to summarize critically and to present adequately the current knowlege concerning the chemistry, industrial production, physiology, biochemistry, estimation, occurrence, deficiency effects, pharmacology, and requirements of each vitamin. Since this is essentially a reference work, some repetition of information in the various sections of each chapter is unavoidable and even desirable.

Neither the clinical manifestations of vitamin deficiencies nor their treatments have been presented in detail, since they are adequately covered in other publications. For the same reason, the methods of vitamin assay are discussed but briefly.

Special emphasis has been given to the chemistry and physiology of the vitamins. This compelled the omission of historical material, except where it bears importantly on current knowledge. An extensive bibliography has been included so that the student can readily consult original material.

The vitamins are presented alphabetically because there is no biological reason why they should be arranged otherwise.

The editors want to express their appreciation to the authors for their scientific devotion and to the publisher for his unending patience. They will feel repaid for their labors if this work contributes to a better understanding of the role of vitamins and stimulates further research, for it has been said truly:

> *Wisdom is the principal thing; therefore get wisdom; and with all thy getting get understanding.*—(Proverbs IV.7)

W. H. Sebrell, Jr.
Robert S. Harris

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I. Nomenclature and Formulas

ROBERT S. HARRIS

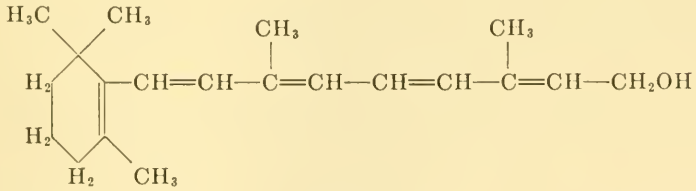
Accepted names: Vitamin A
Neovitamin A
Axerophthol (vitamin A_1)
Carotene

Obsolete names: Fat-soluble A
Biosterol
Ophthalamin
Anti-infective vitamin

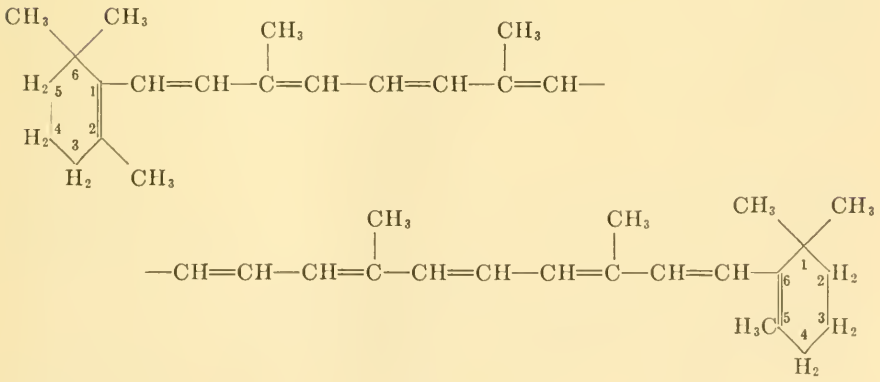
Empirical formulas: Vitamin A: $C_{20}H_{30}O$
β-Carotene: $C_{40}H_{56}$

Chemical name: 3,7-Dimethyl-9-(2,6,6-trimethyl-2-cyclohexen-1-yl)-2,4,6,8-nonatetraen-1-ol

Structures:



Vitamin A



β-Carotene

II. Chemistry and Industrial Preparation

NICHOLAS A. MILAS

The chemistry of vitamin A and that of the closely related carotenoid pigments which act as precursors of this vitamin has been the subject of numerous investigations during the past three decades. Much information of lasting value has accumulated, and an attempt will be made to summarize it in the following sections.

A. THE ANTIXEROPHTHALMIC PROVITAMINS

1. Introduction

The provitamins A are carotenoid pigments[1] which are converted to vitamin A *in vivo*, usually in the intestinal tract of animals,[2, 3] by some as yet unknown mechanism, or *in vitro* by careful oxidative degradation.[4-7] Chemically, provitamin A carotenoids belong to a class of organic compounds known as polyenes which are built up of isoprene units, the most common of which contain eight such units or forty carbon atoms. Carotenoids are, as a rule, hydrocarbons, but some are found to contain hydroxyl, carbonyl, and oxirane or epoxide groups.

There are some well-known characteristics common to all provitamin A carotenoids: (1) all are crystalline solid pigments; (2) in all of them one finds the arrangement of isoprene units reversed in the center of the molecule so that the methyl groups occupy the 1,6 instead of the 1,5 position, and it is this arrangement which makes it possible for the cleavage to occur in the center, thereby producing vitamin A; (3) all contain a large number of carbon-carbon double bonds, most of which are conjugated and are thereby responsible for the production of color and the characteristic absorption spectra of these molecules; (4) all contain at least one trimethylcyclohexenyl or β-ionone ring, usually in conjugation with the open carbon chain double bonds; (5) although it is now well known that all naturally occurring provitamin A carotenoids have the all-*trans* configuration, they

[1] P. Karrer and E. Jucker, Carotenoids (trans. and revised by E. A. Braude). Elsevier Publishing Co., New York, 1950.

[2] (a) E. L. Sexton, J. W. Mehl, and H. J. Deuel, Jr., *J. Nutrition* **31**, 299 (1946); (b) F. H. Mattson, J. W. Mehl, and H. J. Deuel, Jr., *Arch. Biochem.* **15**, 65 (1947); (c) C. E. Wiese, J. W. Mehl, and H. J. Deuel, Jr., *ibid.*, **15**, 75 (1947); A. L. S. Cheng and H. J. Deuel, Jr., *J. Nutrition* **41**, 619 (1950).

[3] S. Y. Thompson, J. Ganguly, and S. K. Kon, *Brit. J. Nutrition* **3**, 50 (1949).

[4] R. F. Hunter and N. E. Williams, *J. Chem. Soc.* **1945**, 554.

[5] G. C. L. Goss and W. D. McFarlane, *Science* **106**, 375 (1947).

[6] N. L. Wendler, C. Rosenblum, and M. Tishler, *J. Am. Chem. Soc.* **72**, 234 (1950).

[7] P. Meunier, J. Jouanneteau, and G. Zwingelstein, *Compt. rend.* **231**, 1170 (1950).

are capable of existing in more than one stereochemical form. Changes of this type bring about changes not only in adsorption affinity and absorption spectra but also in biological activity. Table I shows some of the well-known naturally occurring provitamin A carotenoids together with their important sources, physical properties, and relative physiological activity.

2. Isolation of Provitamin A Carotenoids

a. General Procedure

Well-dried and well-ground materials of either plant or animal origin are extracted in large vessels, percolators, or Soxhlet apparatus by any of the well-known solvents such as low-boiling saturated hydrocarbons, benzene, ether (peroxide-free), chloroform, trichloroethylene, ethanol, or acetone. Extraction is carried out at room temperature in an atmosphere of carbon dioxide or nitrogen. The extracts are then concentrated under a reduced pressure and the concentrates saponified at 60 to 70° with a 5 to 10% methanolic potassium or sodium hydroxide. Water is then added, and the non-saponifiable portion extracted thoroughly with low-boiling petroleum ether. The petroleum ether extract is then partitioned with an equal volume of methanol. Carotenoids which go into the methanolic layer are known as hypophasic and usually contain groups such as hydroxyl, carbonyl, or oxirane, and those which remain in the hydrocarbon layer are known as epiphasic and are usually hydrocarbons such as α-, β-, γ-, or δ-carotenes.

The epiphasic carotenoids have somewhat small differences in molecular structure and are therefore difficult to purify by the well-known classical methods. They show, however, strong selective adsorption on various solid adsorbents such as alumina, Al_2O_3, calcium hydroxide, $Ca(OH)_2$, calcium carbonate, $CaCO_3$, and zinc carbonate, $ZnCO_3$, and can therefore be separated in the pure state by the chromatographic adsorption method first introduced by Tswett.[8] (See also general references on chromatography.[1, 9])

[8] M. Tswett, *Ber. deut. botan. Ges.* **24**, 316, 384 (1906); Chromophylls in Plant and Animal World. Warsaw, 1910.

[9] General references on chromatography:
(a) L. Zechmeister and L. Cholnoky, Chromatographic Adsorption (trans. from the 2nd ed. by A. L. Bacharach and F. A. Robinson). John Wiley and Sons, New York, 1941.
(b) H. H. Strain, Chromatographic Adsorption Analysis. Interscience Publishers, New York, 1942.
(c) T. I. Williams, Chromatographic Analysis. Blackie and Son, London, 1947.
(d) E. Lederer, Chromatographic Analysis. Hermann, Paris, 1949.
(e) L. Zechmeister, Progress in Chromatography 1938–1947. Chapman and Hall, London, 1950.
(f) H. G. Cassidy, Chromatographic Analysis. Interscience Publishers, New York, 1951.

TABLE I

PROPERTIES OF NATURALLY OCCURRING PROVITAMINS A

Provitamin A	Formula	Important sources	Crystals	Melting point	[α]°Cd	Absorption maxima, mμ		Double bonds	Relative physiological activity (β-carotene = 100)
						CS_2	$CHCl_3$		
α-Carotene	$C_{40}H_{56}$	Red palm oil, green chestnut leaves, mountain ash berries	Dark-red prisms (benzene-methanol), dark-red prisms or polygons (ether)	187–188° (corr.)	+385° (18°)	511, 478 446	485, 454	11	53
β-Carotene	$C_{40}H_{56}$	Alfalfa, carrots, green leaves, red palm oil, butter	Dark-red plates, dark-violet hexagonal prisms (b.-m.), dark-red rhombic plates (pet. ether)	181–182°, 184° (corr.) (vacuum capil.)	0	520, 484, 540	497, 466	11	100
γ-Carotene	$C_{40}H_{56}$	*Gonocarium pyriforme*, lilies of the valley (leaves)	Dark-red prisms with a blue luster (b.-m.)	178° (corr.) (vacuum capil.)	0	533, 496, 463	509, 475, 446	12	42[a], 27
-Carotene	$C_{40}H_{58}$	*Gonocarium pyriforme*	Orange to red-colored needles	140.4–140.5°	0	520, 490, 457	503, 470, 440	10	Inactive
α-Carotene monoepoxide	$C_{40}H_{56}O$	Blossoms of various plants (*Tragopogon pratensis*, *Ranunculus acer*)	Reddish-yellow plates (b.-m.)	175° (vacuum capil.)	?	503, 471	483, 454	11	Active
Cryptoxanthin (3-hydroxy β-carotene)	$C_{40}H_{56}O$	Yellow corn, egg yolk, green grass, butter	Lustrous prisms (b.-m.)	169° (corr.) (vacuum capil.)	0	519, 483, 452	497, 463, 433	11	57
Mutatochrome (citroxanthin) (β-carotene oxide ?)	$C_{40}H_{56}O$	Orange peels	Yellow-orange leaflets (b.-m.)	163–164° (vacuum capil.)		4895, 459	469, 438	—	Active
Myxoxanthin (3-keto α-carotene)	$C_{40}H_{64}O$	Blue-green algae	Deep-violet prisms (pyridine-methanol)	168–169°	0	488	473	12	Active
Aphanin (3-keto β-carotene)	$C_{40}H_{54}O$	Blue-green algae	Blue-black leaflets (b.-m.)	176° (corr., b.-m.), 180° (corr., b.-pet. ether)	0	5335, 494	504, 474	11 + 1	50
Echinenone	$C_{40}H_{58}O$	Sea urchin	Violet needles with metallic luster (pet. ether)	178–179° (b.-m.)		520, 488		—	Active
Torularhodin	$C_{37}H_{48}O_2$	Red yeast, *Torula rubra*	Fine red needles (methanol-ether)	201–203° (dec.)		582, 541 502	554, 515 (483)	12	Active
Aphanicin	$C_{80}H_{106}O_3$ (?)	*Aphanizomenon flosaquae*	Red-violet prismatic needles (b.-m.)	190° (corr., b.-m.), 195° (b.-pet. ether)		533, 494	504, 474	12	25

[a] *Ex* pro-γ-carotene by iodine catalysis.

Further purification is accomplished by low temperature fractionation from hydrocarbon solvents.

b. Industrial Preparation

The most widely distributed provitamin A carotenoid in nature is β-carotene, and a number of processes have been developed recently for the large-scale isolation of this carotenoid.[10-12] Certain dry vegetable leaf wastes are rich sources of this carotene. For example, dry spinach, beet, carrot, turnip, kale, and broccoli leaf wastes contain an average of 300 to 700 γ of carotene per gram. Table II shows some of the important industrial sources of β-carotene.[11]

TABLE II

IMPORTANT INDUSTRIAL SOURCES OF β-CAROTENE

Source	Milligrams per pound	
	Fresh	Dry
Carrots	9–11	410
Sweet potatoes	14	64
Alfalfa	28	118
Clover		153
Barley	21	140
Rye	38	203
Sweet clover	15	89
Wheat	20	118

In one[10] of the recent industrial methods dry leaf wastes were ground in a mill to 30–40 mesh and then extracted in a large Soxhlet apparatus of 4.5 kg. (10 lb.) capacity, using low-boiling saturated hydrocarbons (Skellysolve F, b. 35 to 59°). Purification of the extracts was accomplished by one of two methods. The carotene was adsorbed from the extracts directly on magnesia[13] from which it was eluted with a mixture of hexane (Skellysolve B) + 5% acetone. About 85% of the carotene relatively free from chlorophyll and xanthophyll was obtained by this method. A second method was recommended in which the hydrocarbon extracts were mixed with 95% ethanol containing 5% potassium hydroxide and the mixture was refluxed for about one-half hour. Sufficient water was then added to make the alcohol concentration 80%, thereby causing the separation of the layers. The upper layer contained all the carotene in Skellysolve. Chlorophyll and xanthophyll were thus removed and found in the alcohol layer. Adsorption

[10] M. E. Wall, E. G. Kelley, and J. J. Willaman, *Ind. Eng. Chem.* **36**, 1057 (1944).
[11] W. H. Shearon, Jr. and O. F. Gee, *Ind. Eng. Chem.* **41**, 218 (1949).
[12] H. L. Mitchell, W. G. Schrenk, and R. E. Silker, *Ind. Eng. Chem.* **45**, 415 (1953).
[13] H. H. Strain, *J. Biol. Chem.* **105**, 523 (1934).

of the carotene on activated magnesia from the hydrocarbon layer and elution with Skellysolve-acetone mixtures resulted in a recovery of 85 to 95% of the carotene found in the original plant source. For large-scale operations technical hydrated lime was recommended as the adsorbent. With the lime method a concentration of carotene was obtained containing 30,000 to 40,000 I.U. per gram. This was increased to 160,000 to 180,000 I.U. per gram by low temperature fractionations. Fractional crystallization at low temperatures produced a carotene, m.p. 171 to 173° (corr.).

One of the recent large-scale industrial methods[11] uses dehydrated alfalfa meal as the source of carotene. This is extracted with pure hexane by a countercurrent process. The crude extract is concentrated to one-tenth its original volume and the concentrate passed upward through a series of towers containing activated carbon (20 mesh). Chlorophyll is adsorbed at the bottom, xanthophyll next, and carotene at the top of the tower. Continuous washing with hexane separates first the lipoids, then the carotenes, followed by the xanthophyll. To obtain the chlorophyll from the bottom portion of the tower the flow of hexane is reversed. The carotene fraction is further purified by a second chromatographic adsorption. The product thus obtained contains 12 to 20 million I.U. per pound of the concentrate. If purer carotene is desired, the crude concentrate is recrystallized from benzene-isopropanol mixtures. Carotene prepared from alfalfa contains mostly β-carotene and very little of the α isomer.

A very recent modification[12] of the above method makes use of tricalcium phosphate, $Ca_3(PO_4)_2$, to adsorb chlorophyll and xanthophyll almost completely from hexane solutions of these substances together with carotene. Adsorption is accomplished either by stirring tricalcium phosphate (1.2 lb. per gallon of hexane extract) with the extract and then filtering the mixture, or by making use of the radial chromatography method.[14] The efficiency of the recovery of carotene is over 95% with concentrates containing over 300,000 I.U. per gram. Further purification is effected by low temperature fractionation or by a chromatographic adsorption through a magnesia column.

Carotene has also been produced industrially from palm oil[15] and from carrots.[16] However, the carotene produced from these sources contains both the α and β isomers.

3. Chemical Constitution of Provitamin A Carotenoids

a. General

All provitamin A carotenoids are structurally related to one of the three carotenes, α-, β-, or γ-carotene. The elucidation of the chemical structure

[14] P. P. Hopf, *Ind. Eng. Chem.* **39**, 938 (1947).
[15] J. M. Tabor *et al.*, U. S. Pat. 2,440,029 (April 20, 1948).
[16] H. M. Barnett, U. S. Pats. 2,348,443 (May 9, 1944), 2,412,707 (Dec. 17, 1946).

of these carotenes will therefore clarify the chemical structure of all other provitamin A carotenoids. It has already been shown in Table I that these three carotenes are isomeric, having the same empirical composition, $C_{40}H_{56}$. Furthermore, they are isomeric with lycopene, which is devoid of provitamin A activity. Theoretically a hydrocarbon of the above composition should absorb 13 moles of hydrogen to form a saturated hydrocarbon of the empirical composition $C_{40}H_{82}$. Only lycopene[17] yields a hydrocarbon of this composition, showing that it is an open-chain unsaturated hydrocarbon. Catalytic hydrogenation of α-carotene[18] and β-carotene[19] gives the same perhydro compound, $C_{40}H_{78}$, showing the presence of two rings in each carotene. γ-Carotene,[20] on the other hand, absorbs 12 moles of hydrogen to yield perhydro-γ-carotene, $C_{40}H_{80}$, showing the presence of only one ring in the original molecule.

In addition to quantitative hydrogenation and spectroscopic examination of carotenoids, degradative studies are essential for the complete elucidation of their structure. Table III summarizes the products obtained when lycopene and α-, β-, and γ-carotenes are subjected to air oxidation, ozonolysis, hot chromic acid oxidation, alkaline permanganate oxidation, and dry distillation. The last column shows the main literature references.

From the hydrogenation data, absorption spectra, and degradation products shown in Table III, the following conclusions may be drawn regarding the structure of the four carotenoids. Lycopene has thirteen double bonds, eleven of which are conjugated and two isolated. Since 2 moles of acetone are formed on ozonolysis, the isolated double bonds are attached to isopropylidene groups, each of which is separated from the conjugated system by a saturated residue which leads to the formation of succinic acid. Since γ-carotene gives, on degradative oxidation, acetone and geronic and succinic acids, it must have one trimethylcyclohexenyl nucleus similar to that present in β-ionone and an open chain similar to that present in lycopene. β-Carotene has eleven conjugated double bonds and two trimethylcyclohexenyl rings similar to that present in β-ionone. α-Carotene likewise has eleven double bonds, but its absorption spectrum, optical activity, and degradation products indicated that one of the double bonds is isolated in a trimethylcyclohexenyl ring similar to that present in α-ionone. The other ring is of the β-ionone type. Since the provitamin A activity depends in part upon the presence of the β-ionone nucleus, β-carotene which has two such nuclei shows twice as much biological activity as

[17] P. Karrer and R. Widmer, *Helv. Chim. Acta* **11**, 751 (1928).
[18] (a) J. H. C. Smith, *J. Biol. Chem.* **102**, 157 (1932).
(b) R. Kuhn and E. F. Moller, *Z. angew. Chem.* **47**, 145 (1934).
[19] L. Zechmeister, L. Cholnoky, and V. Vrabély, *Ber.* **61**, 566 (1928); **66**, 123 (1935).
[20] R. Kuhn and H. Brockmann, *Ber.* **66**, 407 (1933).

α- or γ-carotene. Lycopene, which is a straight-chain carotenoid, shows no provitamin A activity.

TABLE III

DEGRADATION PRODUCTS OF LYCOPENE AND α-, β-, AND γ-CAROTENES

Carotenoid	O_2 (air)	O_3	CrO_3 (hot)	$KMnO_4$ (alkaline)	Distillation (dry)	References
Lycopene (optically inactive)		2 moles of acetone, succinic acid	6 moles CH_3COOH	Succinic and acetic acids	Toluene, *m*-xylene	21–23
α-Carotene (optically active)	α-Ionone, β-ionone	Geronic acid, isogeronic acid	6 moles CH_3COOH	α,α-Dimethylglutaric acid	2,6-Dimethyl-naphthalene	19, 24, 25
β-Carotene (optically inactive)	β-Ionone	Geronic acid	6 moles CH_3COOH	Geronic, α,α-dimethylglutaric, α,α-dimethylsuccinic, and α, α-dimethylmalonic acids	2,6-dimethyl naphthalene and *m*-xylene	19, 21d, 22, 24c, 26–30
γ-Carotene (optically inactive)	β-Ionone	Geronic acid, acetone	6 moles CH_3COOH	Geronic and succinic acids	2,6-Dimethyl-naphthalene	20, 31, 32

The formation of acetic acid as one of the degradation products indicates[21d, 32] the presence of unsaturated groupings of the type $=\overset{\displaystyle H}{\overset{|}{C}}-\underset{\displaystyle CH_3}{\underset{|}{C}}=$.

[21] P. Karrer and coworkers, *Helv. Chim. Acta* **11**, (a) 751, (b) 1201 (1928); **12**, (c) 285 (1929); **13**, (d) 1084 (1930); **14**, (e) 43 (1931).

[22] R. Pummerer, L. Rebmann and W. Reindel, *Ber.* **62**, 1411 (1929).

[23] R. Kuhn and A. Winterstein, *Ber.* **65**, 1873 (1932).

[24] P. Karrer and coworkers, *Helv. Chim. Acta* **14**, (a) 614, (b) 833, (c) 1033 (1931); **15**, (d) 490, (e) 1158 (1932); **16**, (f) 975 (1933); **17**, (g) 417, (h) 1169 (1934); **18**, (i) 25 (1933).

[25] R. Kuhn and E. Lederer, *Ber.* **64**, 1349 (1931).

[26] R. Pummerer and L. Rebmann, *Ber.* **61**, 1099 (1928).

[27] P. Karrer and coworkers, *Helv. Chim. Acta* **12**, 1142 (1929); **13**, 1084 (1930); **14**, 1033 (1931).

[28] R. Kuhn, A. Winterstein, and L. Karlovitz, *Helv. Chim. Acta* **12**, 64, 904 (1929); *Ber.* **64**, 1732 (1931).

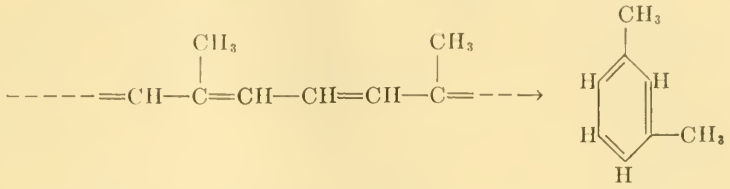
[29] H. H. Strain, *J. Biol. Chem.* **102**, 137 (1931).

[30] R. Kuhn and A. Winterstein, *Ber.* **65**, 1873 (1932); **66**, 429, 1733 (1933).

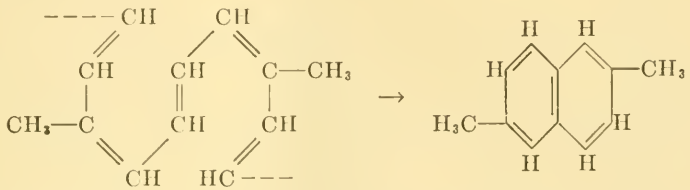
[31] G. MacKinney, *J. Biol. Chem.* **112**, 421 (1935).

[32] R. Kuhn and H. Roth, *Ber.* **66**, 1274 (1933).

The thermal decomposition of these carotenoids leads to the production of *m*-xylene[33, 34] and 2,6-dimethylnaphthalene.[30] *m*-Xylene is assumed to be derived from an unsaturated fragment;



whereas 2,6-dimethylnaphthalene is derived from the central portion of the conjugated chain.



On the basis of these conclusions the structure of lycopene and that of the naturally occuring provitamin A carotenoids is given in Table IV.

The structure of δ-carotene mentioned in Table I is not definitely known, although a tentative structure has been proposed recently by Porter and Murphey.[35] The structure of echinenone is also in doubt, although Lederer[36] suggested that it may be identical with myxoxanthin. Aphanicin may be considered as two molecules of aphanin joined by means of an oxygen bridge.

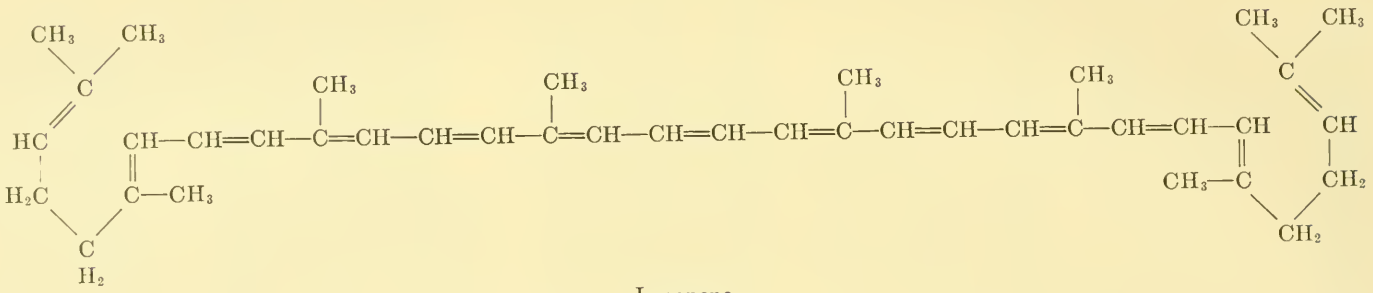
b. Stereochemical Configuration of Provitamin A Carotenoids

It has already been shown that provitamin A carotenoids contain several conjugated double bonds in an open chain. Since the terminal double bonds in the cyclohexenyl rings are fixed, only the double bonds in the open chain are subject to geometrical isomerism. Theoretically β-carotene with nine double bonds in an open chain should exist in 512 *cis-trans* isomers. However, on certain theoretical grounds Pauling[37] and later Zechmeister[38] have shown that some of the double bonds are sterically hindered and can exist only in the *trans* form. Furthermore, the symmetry of the structure of a

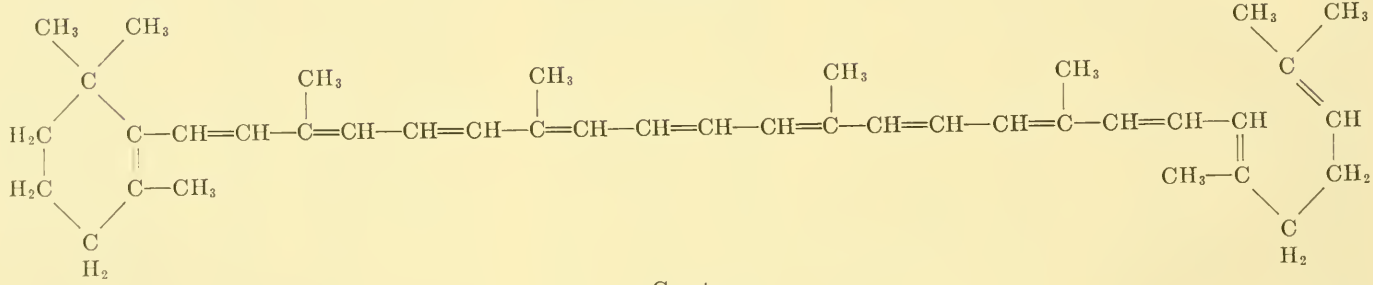
[33] R. Kuhn and A. Winterstein, *Helv. Chim. Acta* **11**, 427 (1928).
[34] L. Zechmeister and L. Cholnoky, *Ann.* **478**, 95 (1930).
[35] J. W. Porter and M. M. Murphey, *Arch. Biochem. and Biophys.* **32**, 21 (1951).
[36] P. Karrer and E. Jucker, Carotenoids (trans. and revised by E. A. Brande), p. 325. Elsevier Publishing Co., New York, 1950.
[37] L. Pauling, *Fortschr. Chem. org. Naturstoffe* **3**, 203 (1939).
[38] L. Zechmeister, *Chem. Revs.* **34**, 267 (1944).

TABLE IV

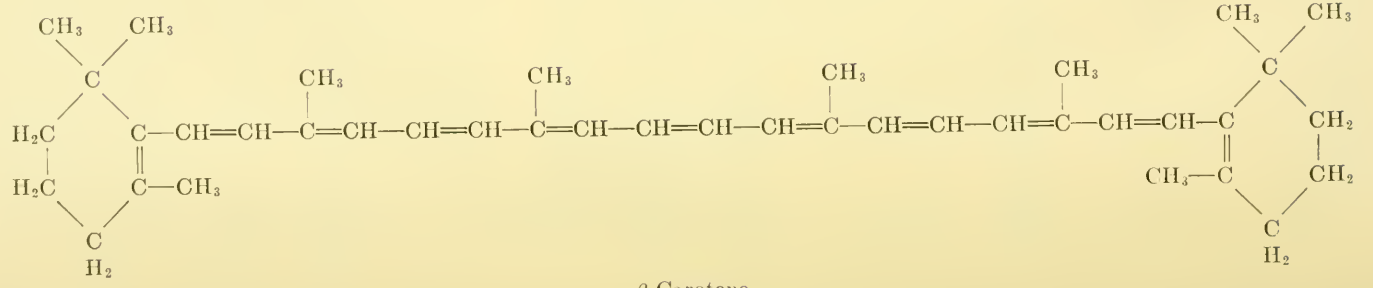
THE STRUCTURE OF LYCOPENE AND OF THE NATURALLY OCCURRING PROVITAMIN A CARTENOIDS



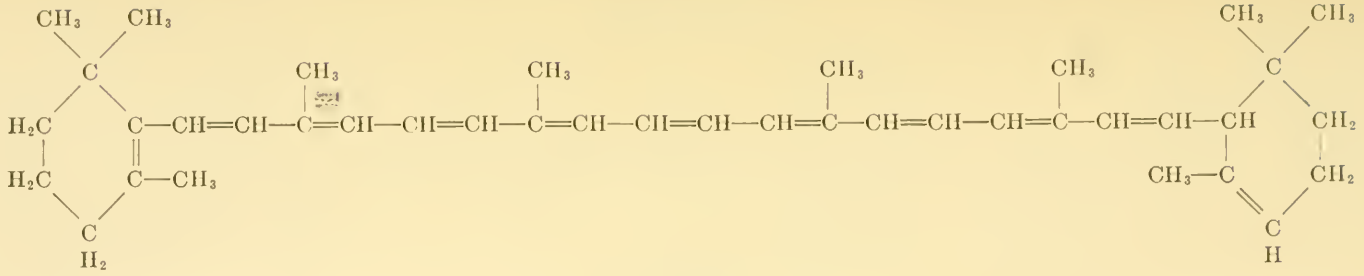
Lycopene



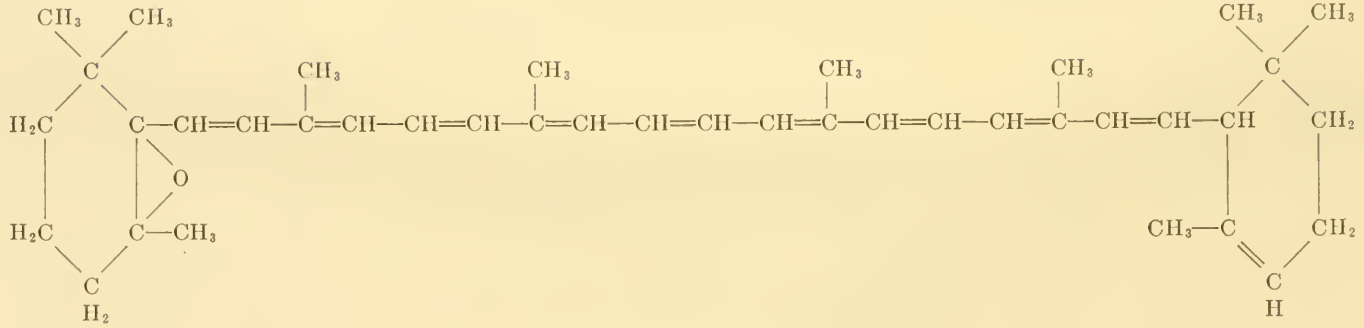
γ-Carotene



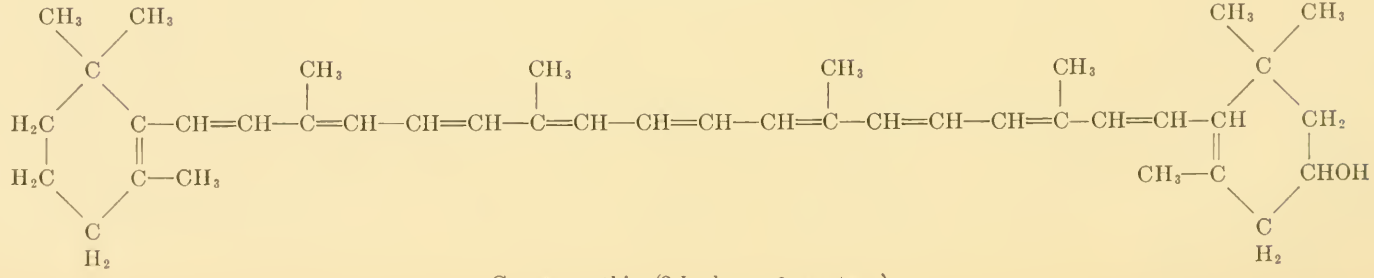
β-Carotene



α-Carotene

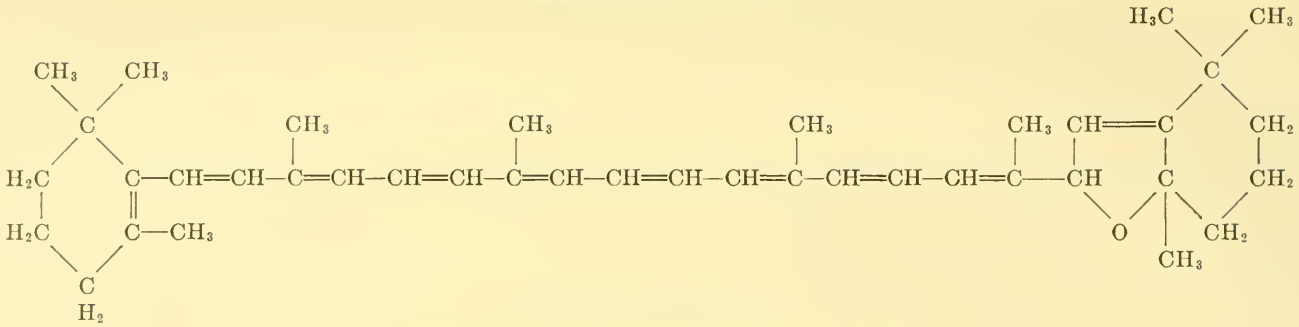


α-Carotene monoepoxide

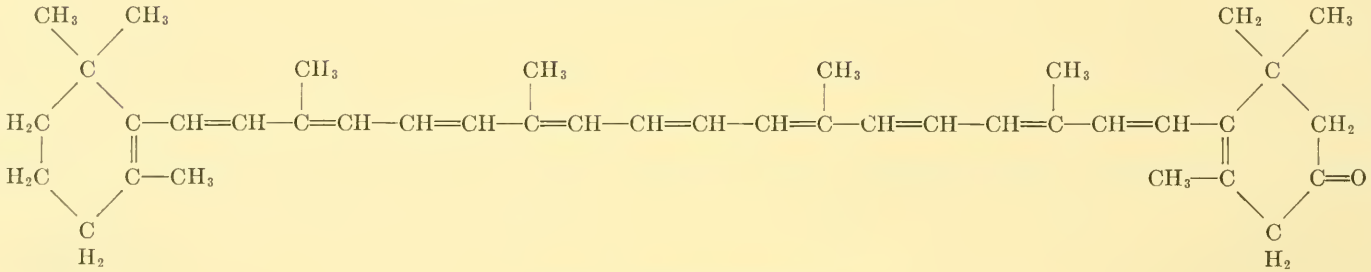


Cryptoxanthin (3-hydroxy β-carotene)

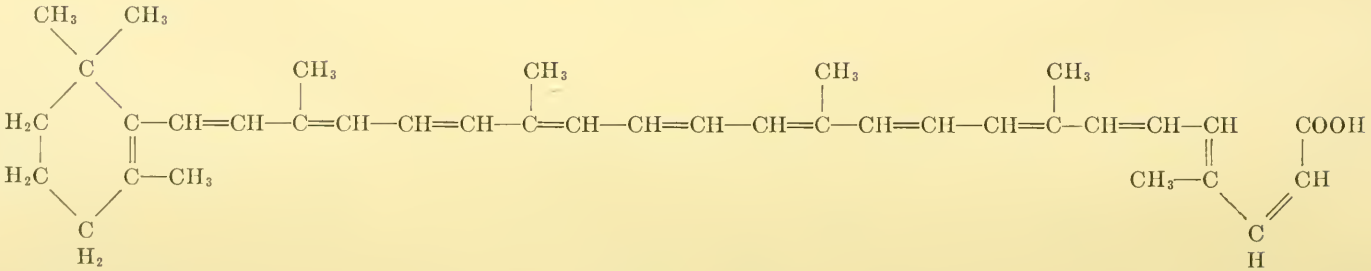
TABLE IV—*Continued*



Mutatochrome (citroxanthin) (β-carotene oxide?)



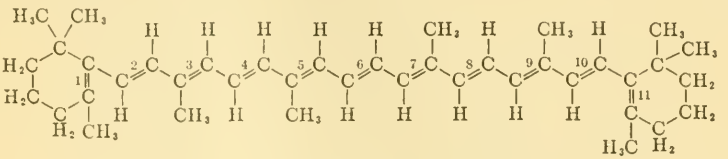
Aphanin (3-keto β-carotene) (?)



Torularhodin (?)

given carotenoid limits its number of *cis-trans* isomers. These restrictions limit the total possible isomers for β-carotene (sym.) to 20, for α-carotene (unsym.) to 32, for γ-carotene (unsym.) to 64, and for cryptoxanthin (unsym.) to 32.

It has now been well established[39] that most of the naturally occurring provitamin A carotenoids exist in an all-*trans* configuration. The all-*trans* configuration for β-carotene is shown in structure I in which double bonds



I All-*trans*-β-carotene

3, 5, 6, 7, and 9 are capable of existing in both *cis* and *trans* isomers, and with the exception of double bonds 1 and 11, which are fixed, 2, 4, 8, and 10 are sterically hindered and exist only in *trans* form. Table V gives a set of twenty possible isomers of β-carotene.

TABLE V

POSSIBLE *cis-trans* ISOMERS OF β-CAROTENE

Isomer	Configuration	Isomer	Configuration
I	All-*trans*	XI	3,5,6-Tri-*cis*
II	3-*cis*	XII	3,5,7-Tri-*cis*
III	5-*cis*	XIII	3,5,9-Tri-*cis*
IV	6-*cis*	XIV	3,6,7-Tri-*cis*
V	3,5-Di-*cis*	XV	3,6,9-Tri-*cis*
VI	3,6-Di-*cis*	XVI	5,6,7-Tri-*cis*
VII	3,7-Di-*cis*	XVII	3,5,6,7-Tetra-*cis*
VIII	3,9-Di-*cis*	XVIII	3,5,6,9-Tetra-*cis*
IX	5,6-Di-*cis*	XIX	3,5,7,9-Tetra-*cis*
X	5,7-Di-*cis*	XX	3,5,6,7,9-Penta-*cis*

The all-*trans* configuration of provitamin A carotenoids is preserved only when they are freshly extracted from natural sources or kept in the crystalline form. In solution some of the sterically unhindered double bonds slowly change configuration, and a mixture of several *cis-trans* isomers is produced. This change can be brought about more rapidly when the solutions are refluxed in the dark or exposed to light in the presence of catalytic amounts of iodine. The change can be easily detected spectroscopically, since there is a decrease in color intensity and the absorption maxima shift from the visible toward the ultraviolet region of the spectrum. In

[39] L. Zechmeister, *Vitamins and Hormones* **7**, 57 (1949).

addition, a "*cis*-peak" appears in the region of about 340 mμ, the height of which depends upon the number of *cis* isomers. These changes are illustrated for α-, β-, and γ-carotenes in Figs. 1, 2, and 3. Since the adsorption affinities

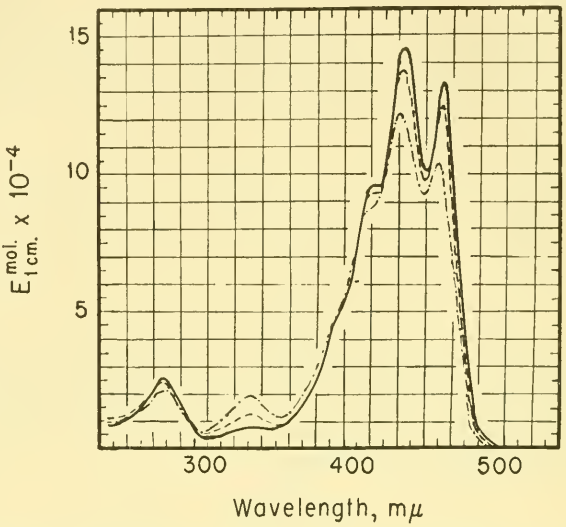


FIG. 1. Molecular extinction curves of α-carotene in hexane: ——, fresh solution of the all-*trans* form; — — —, mixture of stereoisomers after refluxing in the dark for 45 minutes; —·—, mixture of stereoisomers after iodine catalysis in light at room temperature. [From *J. Am. Chem. Soc.* **65**, 1522 (1943).]

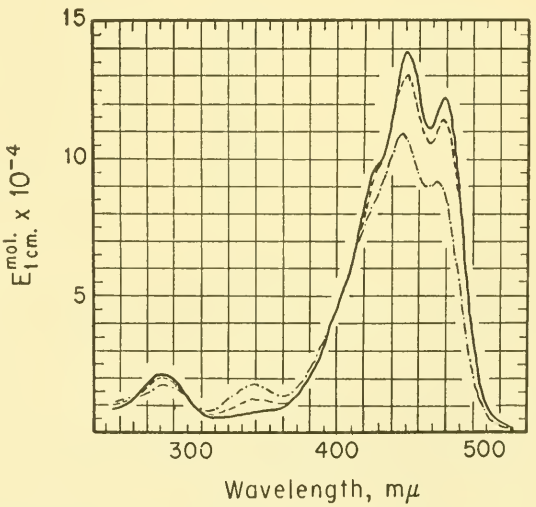


FIG. 2. Molecular extinction curves of β-carotene in hexane: ——, fresh solution of the all-*trans* form; — — —, mixture of stereoisomers after refluxing in the dark for 45 minutes; —·—, mixture of stereoisomers after iodine catalysis in light at room temperature. [From *J. Am. Chem. Soc.* **65**, 1523 (1943).]

of the various isomers are different, they can be separated chromatographically.

The change from an all-*trans* to a *cis-trans* configuration of a provitamin A molecule usually causes a decrease in the biological potency of the molecule. This may be interpreted as being due to the change of shape of the molecule which may not fit into the biological systems that cause the breakdown of the provitamin A into vitamin A. Usually the all-*trans* forms of provitamins A show a higher biological potency than their geometrical

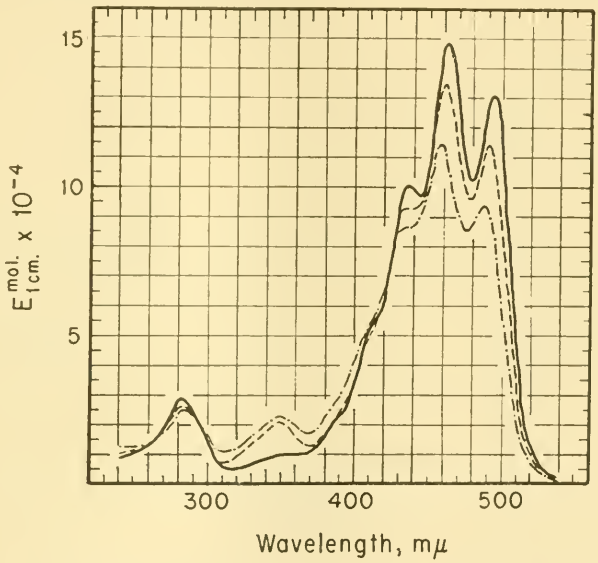


FIG. 3. Molecular extinction curves of γ-carotene in hexane: ——, fresh solution of the all-*trans* form; — — —, mixture of stereoisomers after refluxing in the dark for 45 minutes, —·—, mixture of stereoisomers after iodine catalysis in light at room temperature. [From *J. Am. Chem. Soc.* **65**, 1941 (1943).]

isomers. Table VI gives the relative biological potencies of some of the well-known stereoisomeric provitamin A carotenoids.

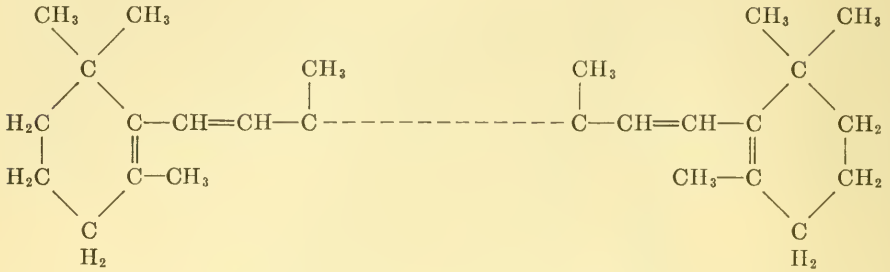
4. Biologically Active Derivatives of Provitamins A

Provitamin A carotenoids can, under specified conditions, be converted into certain derivatives which retain their biological activity. For example, α- and β-carotenes react with cold concentrated hydrogen iodide to give, among other products, 5,6-dihydro-α- and β-carotenes.[40] In this case one of the trimethylcyclohexenyl rings has become fully hydrogenated. With monoperphthalic acid[41, 42] both α- and β-carotenes form monoepoxides

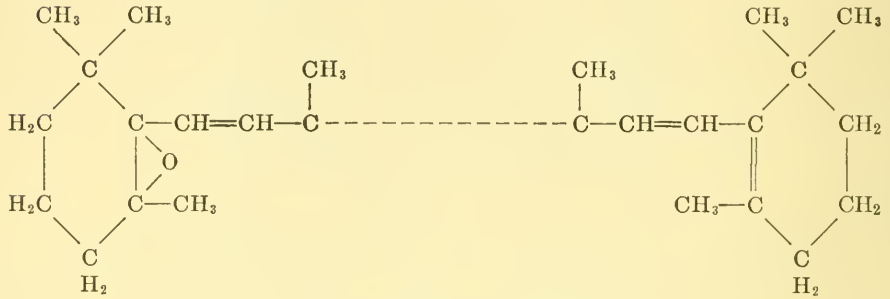
[40] A. Polgár and L. Zechmeister, *J. Am. Chem. Soc.* **65**, 1528 (1943).

[41] P. Karrer and coworkers, *Helv. Chim. Acta* **28**, 300, 427, 471, 474, 717, 1143, 1156 (1945); **30**, 531, 536 (1947).

which are highly labile and rearrange in the presence of traces of acids to monofuranoxides. These changes may be illustrated as follows:

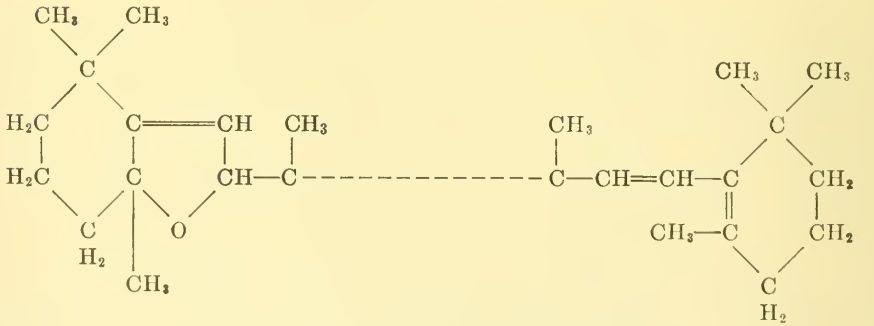


β-Carotene (skeleton) + monoperphthalic acid



XXI β-Carotene monoepoxide

H+



XXII β-Carotene monofuranoxide (mutatochrome)

Similarly β-carotene forms a diepoxide (XXIII) which rearranges on one side to give luteochrome (XXIV, β-carotene monoepoxide monofuranoxide) or on both sides to give aurochrome (XXV, β-carotene difuranoxide). α-Carotene does not form a diepoxide. When β-carotene is carefully treated

[42] P. Karrer, *Fortschr. Chem. org. Naturstoffe* **5**, 1 (1948).

with dilute (0.1 *N*) chromic acid, 5,6-dihydroxy β-carotene is formed. With a larger amount of chromic acid, one of the trimethylcyclohexenyl rings is opened to form semi-β-carotenone (XXVI) which can lose water easily to give anhydro-β-carotenone (XXVII). Karrer and his associates oxidized carefully β-carotene with potassium permanganate and destroyed one of the trimethylcyclohexenyl rings without affecting the other ring or the con-

TABLE VI

RELATIVE POTENCIES OF STEREOISOMERIC PROVITAMIN A CAROTENOIDS IN THE RAT (ZECHMEISTER)

Stereoisomeric set	Current name	Configuration (probable)	Biopotency: In % of that of the all-*trans* form of the set	Biopotency: In % of the potency of all-*trans* β-carotene
α-Carotene	α-Carotene	All-*trans*	100	53
	Neo-α-carotene U	3- or 9-Mono-*cis*	25	13
	Neo-α-carotene B	3,6- or 3,7-Di-*cis*	30	16
β-Carotene	β-Carotene	All-*trans*	100	100
	Neo-β-carotene U	3-Mono-*cis*	38	38
	Neo-β-carotene B	3,6- or 3,7-Di-*cis*	53	53
γ-Carotene	γ-Carotene[a]	All-*trans*	100	27
	γ-Carotene[b]	All-*trans*		42
	Neo-γ-carotene P	3-Mono-*cis*	70	19
	Mixture	Central mono-*cis*	60	16
	Pro-γ-carotene	Poly-*cis* (penta-*cis*)	160	44
Cryptoxanthin	Cryptoxanthin	All-*trans*	100	57
	Neocryptoxanthin U	3-Mono-*cis*	45	27
	Neocryptoxanthin A	Central mono-*cis*	74	42

[a] *Ex Pyracantha.*

[b] *Ex* pro-γ-carotene by iodine catalysis.

jugated system. Thus, they produced 2-apocarotenal (XXVIII) which was reduced with aluminum isopropoxide to 2-apocarotenol. Using the same method but different amounts of potassium permanganate, the same workers oxidized β-carotene at the fourth double bond and obtained 4-apocarotenal (XXIX), which was also reduced to 4-apocarotenol. All these products were found to be biologically active. Table VII lists these provitamins A together with some of their physical properties.

It has also been known for some time[49] that both α- and β-carotenes add iodine to form the corresponding diiodides, which are biologically active.

In certain cases biologically inactive carotenoids have been converted to

biologically active provitamin A products by chemical treatment. Both xanthophyll and zeaxanthin are biologically inactive, but when treated with phosphorus tribromide, biologically active products are obtained.[50]

TABLE VII

BIOLOGICALLY ACTIVE DERIVATIVES OF PROVITAMIN A CAROTENOIDS

Derivative	Formula	M.p., C.°	Absorption maxima (benzene), mμ	References
5,6-Dihydro-α-carotene	$C_{40}H_{58}$	202–203	483.5, 453.5	40
5,6-Dihydro-β-carotene	$C_{40}H_{58}$	164	489, 458	40
α-Carotene monoepoxide	$C_{40}H_{56}O$	175	484, 455	41, 42
β-Carotene monoepoxide	$C_{40}H_{56}O$	160	492, 460	41, 42
β-Carotene monofuranoxide (mutatochrome)	$C_{40}H_{56}O$	163–164	470, 440	41–43
β-Carotene diepoxide	$C_{40}H_{56}O_2$	184	485, 456	41, 42
β-Carotene difuranoxide (aurochrome)	$C_{40}H_{56}O_2$	185	457, 428 (CS_2)	41, 42
β-Carotene monoepoxide monofuranoxide	$C_{40}H_{56}O_2$	176	482, 451 (CS_2)	41, 42
5,6-Dihydroxy β-carotene	$C_{40}H_{56}O_2$	184	489, 487, 428	44
Semi-β-carotenone	$C_{40}H_{56}O_2$	118–119	518, 486, 458	44, 45
Anhydrosemi-β-carotenone	$C_{40}H_{54}O$	177	528, 490, 459	46
β-Apo-2-carotenal	$C_{30}H_{40}O$	139	525, 490 (CS_2)	47
β-Apo-2-carotenol	$C_{30}H_{42}O$	145	486, 456 (CS_2)	48
β-Apo-4-carotenal	$C_{25}H_{34}O$		460 (diffuse)	47, 48
β-Apo-4-carotenol	$C_{25}H_{36}O$			47, 48
α-Carotene diiodide	$C_{40}H_{56}I_2$			49
β-Carotene diiodide	$C_{40}H_{56}I_2$			49
Xanthophyll + PBr_3				50
Zeaxanthin + PBr_3				50

Kitol,[51-55] which belongs to the last group, is not only devoid of vitamin

[43] H. von Euler, P. Karrer, and O. Walker, *Helv. Chim. Acta* **15**, 1507 (1932).
[44] R. Kuhn and H. Brockmann, *Ber.* **67**, 1408 (1934); *Ann.* **516**, 99 (1935).
[45] R. Kuhn and H. Brockmann, *Ber.* **66**, 1319 (1933).
[46] R. Kuhn and H. Brockmann, *Ann.* **516**, 113, 122 (1935).
[47] P. Karrer and H. Solemssen, *Helv. Chim. Acta* **20**, 682 (1937).
[48] H. von Euler, P. Karrer, and H. Solemssen, *Helv. Chim. Acta* **21**, 211 (1938).
[49] P. Karrer, H. von Euler, H. Solemssen, and O. Walker, *Helv. Chim. Acta* **17**, 417, 1169 (1934).
[50] H. von Euler, P. Karrer, and A. Zubrys, *Helv. Chim. Acta* **17**, 24 (1934).
[51] H. Pritchard, H. Wilkinson, J. R. Edisbury, and R. A. Morton, *Biochem. J.* **31**, 258 (1937).
[52] H. Willstaedt and H. B. Jensen, *Nature* **143**, 474 (1939).
[53] H. Kringstad and J. Lee, *Tidskr. Kjemi Bergvesen Met.* **1**, 82 (1941).
[54] N. D. Embree and E. M. Shantz, *J. Am. Chem. Soc.* **65**, 910 (1943).
[55] F. B. Clough, H. M. Kascher, C. D. Robeson, and J. G. Baxter, *Science* **105**, 436 (1947).

A activity but acts physiologically to detoxify excessively high stores of vitamin A. It has been isolated from whale (0.8%), shark (0.8%), and dogfish (0.08%) liver oils. It crystallizes from methanol in colorless elongated prisms, m.p. 88 to 90°, $E^{1\%}_{1\,cm.}$ (290 mμ) 707. It has a composition corresponding to the formula $C_{40}H_{58}(OH)_2$ and forms a dinitrobenzoate, m.p. 200°; two diphenyl benzoates, m.p. 125 to 126° (methyl acetate) and 149 to 150° (acetone), respectively; and an anthraquinone carboxylate, m.p. 195 to 197°. It is also optically active, $[\alpha]^{25°}_{546\ m\mu} = -1.35$ (chloroform). When its palmitate is distilled in a cyclic molecular still (240 to 270°; 0.3 μ), 0.67 to 0.75 mole of vitamin A palmitate is formed per mole of kitol palmitate destroyed. The structure of this interesting carotenoid has not yet been elucidated.

5. Total Synthesis of Provitamin A Carotenoids

Almost simultaneously three groups[56-58] of investigators reported the synthesis of β-carotene.[59] The synthesis of this and other carotenoids was made possible by the discovery of several new reactions and intermediates in the synthesis of vitamin A.[60] One of the intermediates that played an important role in the synthesis of β-carotene was the octenedione (XXXI), which was first synthesized by Mange[61] in the author's laboratory by the action of dimethyl cadmium on *trans*-dihydromuconyl chloride. Since then, other methods[56, 62] have been used for the synthesis of this ketone. The first synthesis[56] of β-carotene made use of this ketone and the Grignard of the acetylenic carbinol (XXX) which was made from β-ionone and propargyl bromide via the Reformatsky reaction. The tetrol XXXII produced was selectively hydrogenated to give the tetrol XXXIII, which was dehydrated to β-carotene with *p*-toluenesulfonic acid in boiling toluene. The yields of β-carotene by this method are rather low, owing to the tendency of the tertiary hydroxyl groups in the carbinol XXX as well as that of the same hydroxyl groups in the subsequent intermediates to undergo an anionic rearrangement to produce a tetrol which does not lead to β-carotene. This objection has been overcome by the second[57] and third methods,[58] which use the intermediates XXXIV and XXXV, respectively.

[56] P. Karrer and C. H. Eugster, *Compt. rend.* **230**, 1920 (1950); *Helv. Chim. Acta* **33**, 1172 (1950).

[57] H. H. Inhoffen, F. Bohlmann, K. Bartram, and H. Pommer, *Chem.-Ztg.* **74**, 285 (1950); *Abhandl. braunschweig. wiss. Ges.* **2**, 75 (1950).

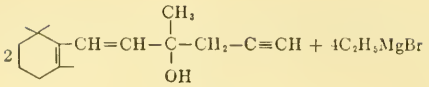
[58] N. A. Milas, P. Davis, I. Belič, and D. A. Fleš, *J. Am. Chem. Soc.* **72**, 4844 (1950).

[59] For a complete review of this subject see H. H. Inhoffen and H. Siemer, *Fortschr. Chem. org. Naturstoffe* **9**, 1 (1952).

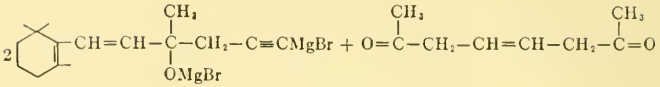
[60] See Section II.B of this chapter.

[61] E. E. Mange, B. S. Thesis, Massachusetts Institute of Technology, 1948.

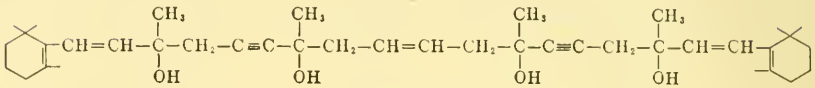
[62] R. Ahmad, F. Sondheimer, B. C. L. Weedon, and R. J. Woods, *J. Chem. Soc.* **1952**, 4089.



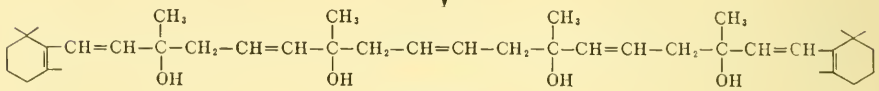
XXX



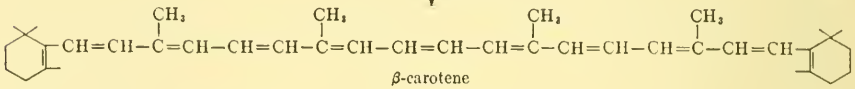
XXXI



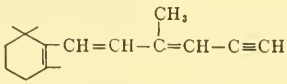
XXXII



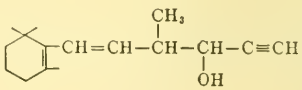
XXXIII



β-carotene

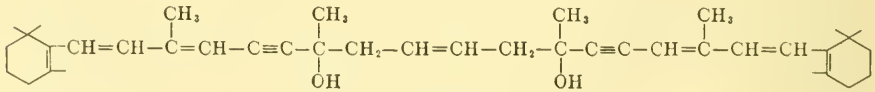


XXXIV



XXXV

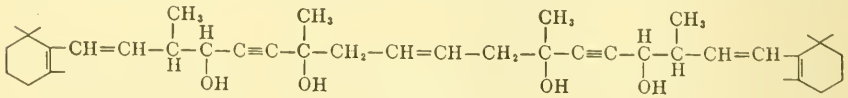
In the second synthesis the lithium derivative of the polyvinyl acetylene XXXIV was condensed with the diketone XXXI to give the glycol XXXVI



XXXVI

which was selectively hydrogenated by means of a poisoned palladium catalyst (Lindlar)[60] to give the corresponding polyolefinic glycol which in turn was dehydrated to give β-carotene.

In the third synthesis the Grignard of the carbinol XXXV was condensed



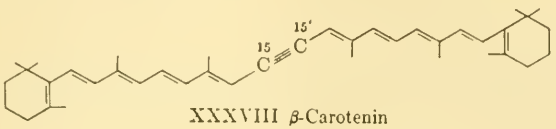
XXXVII

with the diketone XXXI to give the tetrol XXXVII which was selectively hydrogenated to the corresponding polyolefinic tetrol which in turn was dehydrated with pyridine hydrobromide to give β-carotene. In each of the three methods the synthetic product was compared with the natural and the two were found identical. Other synthetic variations of the above method are described by Inhoffen and Siemer.[59]

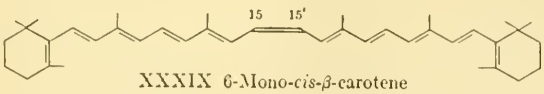
The synthesis of β-carotene opened the way for the synthesis of several carotenoids which are not found in nature. Some of these are briefly described below.

TABLE VIII

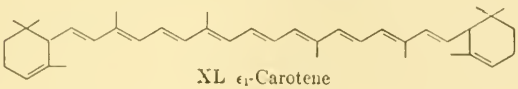
SYNTHETIC CAROTENOIDS UNKNOWN IN NATURE



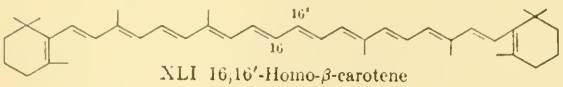
XXXVIII β-Carotenin



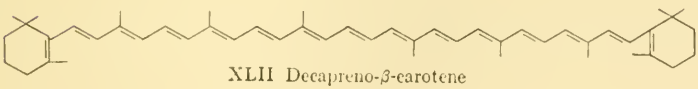
XXXIX 6-Mono-*cis*-β-carotene



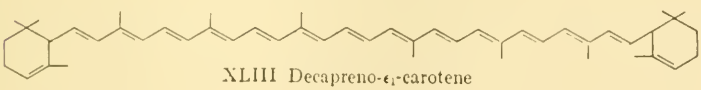
XL ϵ_1-Carotene



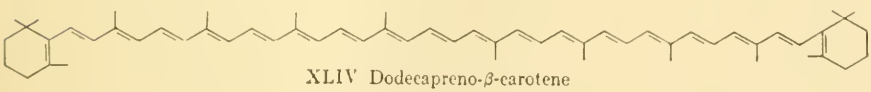
XLI 16,16'-Homo-β-carotene



XLII Decapreno-β-carotene



XLIII Decapreno-ϵ_1-carotene



XLIV Dodecapreno-β-carotene

β-Carotenin (15,15'-dehydro-β-carotene) (XXXVIII)[59] is biologically active in doses of 10 γ.

6-Mono-*cis*-β-carotene (15,15'-mono-*cis*-β-carotene) (XXXIX) was synthesized from β-carotenin by selective hydrogenation using the Lindlar catalyst. This carotenoid is unstable and changes slowly on standing in

diffuse light to all-*trans*-β-carotene. Its biological activity is about one-half that of the all-*trans*-β-carotene.[63]

ϵ_1-Carotene (XL) was synthesized by Karrer and Eugster.[64] It is an isomer of α- and β-carotenes, but it contains two α-ionone rings and was found to be completely devoid of biological activity.

Four higher homologs of β-carotene have also been synthesized: 16,-16′-homo-β-carotene (XLI),[65] biologically active; decapreno-β-carotene (XLII);[66] decapreno-ϵ_1-carotene (XLIII);[67] and dodecapreno-β-carotene (XXLIV).[68] The structural formulas of these carotenoids are given in Table VIII. For further discussion of the synthesis and properties of these carotenoids, the reader is referred to the original literature.

6. Conversion of Provitamin A Carotenoids to Vitamin A

The conversion of provitamins A *in vivo* is a complicated process and is not well understood at present. It has already been mentioned[2, 3] that the conversion probably occurs in the intestinal tract of animals. If the conversion is enzymatic, as it is generally believed,[39] the over-all shape of the provitamin A molecule must be such that it fits the enzyme system. The actual process, however, may be either hydrolytic or oxidative. Karrer *et al.*[69] were the first to suggest that β-carotene is cleaved symmetrically by adding two molecules of water at the 15,15′ double bond to give two molecules of vitamin A. This simple hypothesis was not realized in prac-

$$C_{40}H_{56} + 2H_2O \rightarrow 2C_{20}H_{29}OH$$

tice, since on an equal weight basis β-carotene had always a much lower biological potency than vitamin A. However, recently Koehn[70] has claimed that in the presence of α-tocopherol the fission of β-carotene to vitamin A is accomplished almost quantitatively by the rat.

Theoretically the addition of water to the double bond 15,15′ will not give vitamin A unless the water is first oxidized to hydrogen peroxide which will then add to the double bond in the presence of the enzyme as two hydroxyl groups to give the intermediate 15,15′-dihydroxy β-carotene (XLV). Both hydroxyl groups are allylic and of the same type as the hydroxyl group present in vitamin A. Furthermore, such a dihydroxy caro-

[63] L. Zechmeister, H. J. Deuel, Jr., H. H. Inhoffen, J. Leemann, S. M. Greenberg, and J. Ganguly, *Arch. Biochem. and Biophys.* **36**, 80 (1952).
[64] P. Karrer and C. H. Eugster, *Helv. Chim. Acta* **33**, 1433 (1950).
[65] H. H. Inhoffen, F. Bohlmann, H. J. Aldag, S. Bork, and G. Leidner, *Ann.* **573**, 1 (1951).
[66] P. Karrer and C. H. Eugster, *Helv. Chim. Acta* **34**, 28, 1805 (1951).
[67] P. Karrer, C. H. Eugster, and M. Faust, *Helv. Chim. Acta* **34**, 823 (1951).
[68] P. Karrer and C. H. Eugster, *Helv. Chim. Acta* **34**, 1805 (1951).
[69] P. Karrer, R. Morf, and K. Schöpp, *Helv. Chim. Acta* **14**, 1036, 1431 (1931).
[70] C. J. Koehn, *Arch. Biochem.* **17**, 337 (1948).

tene may easily disproportionate *in vivo* to give one molecule of vitamin A and one molecule of vitamin A aldehyde. The latter could easily be reduced *in vivo* to vitamin A. Such an hypothesis can find ample support in the recent hydroxylation experiments of β-carotene.[4-6]

$$C_{19}H_{27}\overset{15}{C}H{=}\overset{15'}{C}H{-}C_{19}H_{27} + HOOH \xrightarrow{\text{enzyme}} C_{19}H_{27}CH(OH){-}CH(OH){-}C_{19}H_{27}$$

XLV

$$XLV \rightarrow C_{19}H_{27}CH_2OH + C_{19}H_{27}CHO + 2H \rightarrow 2C_{19}H_{27}CH_2OH$$

A slightly different oxidative cleavage has been proposed by Hunter,[71] who assumed that β-carotene is cleaved *in vivo* to give two molecules of vitamin A aldehyde which are subsequently reduced to two molecules of vitamin A.

Attempts to duplicate the cleavage of β-carotene *in vivo* using biochemical methods produced doubtful results.[2b]

For a long time even chemical methods, as shown under Section II. A. 4, failed to cleave β-carotene symmetrically. Using hydrogen peroxide in a chloroform–acetic acid solution, Hunter and Williams[4] were the first to achieve the symmetrical cleavage of β-carotene, although the yields of vitamin A aldehyde obtained were small (0.4 to 0.5%). It had already been known for some time[72] that hydrogen peroxide in non-aqueous solvents causes an efficient hydroxylation of double bonds in the presence of small amounts of osmium tetroxide, so Goss and McFarlane[5] used this method to effect the cleavage of β-carotene. They claimed that a hypothetical intermediate was formed which upon treatment with alkali produced vitamin A directly. This reaction was studied more thoroughly by Wendler *et al.*,[6] who reported the isolation of vitamin A aldehyde, β-ionylidene acetaldehyde, and 2,7-dimethyloctatriendial. They found that the hydroxylation of β-carotene is not entirely symmetrical but takes place most readily at the double bonds which are least substituted and sterically most accessible. The oxidation of β-carotene to give vitamin A aldehyde was also effected with vanadium pentoxide by Meunier *et al.*[7]

B. THE ANTIXEROPHTHALMIC VITAMINS OR VITAMINS A

1. Introduction

The early literature in this field has been ably summarized in two excellent monographs,[73, 74] and no attempt will be made here to treat the subject exhaustively.

[71] R. F. Hunter, *Nature* **158**, 257 (1946).

[72] N. A. Milas and S. Sussman, *J. Am. Chem. Soc.* **58**, 1302 (1936); **59**, 2545 (1937).

[73] H. R. Rosenberg, Chemistry and Physiology of the Vitamins, pp. 37–95. Interscience Publishers, New York, 1942.

It has already been stated in the previous section that the antixerophthalmic vitamins are produced in nature from the corresponding provitamins A. Since the conversion is known to occur in the animal organism only, the vitamins A are entirely of animal origin and are not found in plants. In most cases, but especially in fishes, more than one vitamin A is found in the same species. This is probably due to the ease of interconversion of the various forms in the organism. Structurally, all vitamins A are closely related; some, like vitamin A_1 and neovitamin A, are stereoisomers; others, like vitamin A_2, vitamin A aldehyde or retinene, and anhydrovitamin A_1, are presumably dehydrogenation, oxidation, and dehydration products, respectively, of vitamin A_1. Rehydrovitamin A is formed in the rat by the addition of water to anhydrovitamin A.[75] In addition to vitamins A isolated from natural sources, several synthetic products which do not seem to exist in nature have been prepared and found to exhibit appreciable biological activity. The preparation of an antivitamin A has also been reported recently.[76] These and other developments will now be discussed in the following sections.

2. Vitamin A_1 (Axerophthol)

a. Sources

Vitamin A_1 is the most commonly known and abundant of all the naturally occurring vitamins A. It occurs in all land animals and in sea- and fresh-water fishes. Frequently it occurs together with less common vitamins A. Although this vitamin is present in all organs of the body, skeletal tissues, and blood serum, it is found stored in larger quantities in the liver and viscera. The most important natural source of this vitamin is fish liver oils, and, prior to the appearance of the synthetic vitamin A in the market, all industrial and medicinal vitamin A was obtained from this source. The great scarcity of vitamin A during World War II and its importance in animal nutrition, pharmaceuticals, and fortification of foods, especially margarine, stimulated an extensive search for new sources. Fish liver oils and oils from viscera of numerous species were examined during this period.[77-79] Table IX shows some of the more important sources of vitamin A. The potency shown in the last column includes both vitamin A_1 and neovitamin A. As it may be seen, several of the oils contain also vitamin A_2.

[74] H. Vogel, Chemie und Technik der Vitamine, Erster Band: Die fettlöslichen Vitamine, 3rd ed. (revised by H. Knobloch), pp. 18–150. Ferdinand Enke, Stuttgart, Germany, 1950.

[75] E. M. Shantz, *J. Biol. Chem.* **182**, 515 (1950).

[76] P. Meunier, J. Jounneteau, and R. Ferrando, *Compt. rend.* **230**, 140 (1950); P. Meunier, *Fortschr. Chem. org. Naturstoffe* **9**, 102 (1952).

[77] W. S. Rapson and H. M. Schwartz, *J. Chem. Ind.* **63**, 18 (1944).

[78] U. S. Kini and K. Chidambaram, *J. Chem. Ind.* **66**, 233 (1947).

[79] R. O. Sinnhumber and D. K. Law, *Ind. Eng. Chem.* **39**, 1309 (1947).

TABLE IX

NATURAL SOURCES RICH IN VITAMIN A

Common and scientific name	Source of oil	Oil content, %	Vitamin A, I.U./g. oil
Lancetfish (*Alepisaurus ferox*)	Liver	37.0	5,250
Midshipman (*Porichthys notatus*)	Liver	15.9	6,830
Green sturgeon (*Acipenser acutirostris*)	Liver	24.0	1,610[a]
	Viscera	1.60	8,500[a]
Mackerel shark (*Lamna nasus*)	Liver	63.1	9,000
California pompano (*Perilus simullisimus*)	Liver	8–14	8,000–14,000
Eel pout (*Aprodon cortexianus*)	Liver	4.76	13,760
Pacific hake (*Merluccius productus*)	Liver	32.8	17,260
Thresher shark (*Alopias vulpinus*)	Liver	37–39	1,000–17,500
Great blue shark (*Prionace glauca*)	Liver	37.0	17,700
Three-toothed lamprey (*Entosphenus tridentatus*)	Liver	20–30	12,000–20,000[a]
White sturgeon (*Acipenser transmontanus*)	Liver	9–49	10,000–17,000[a]
	Viscera	2–5.5	10,000–28,000[a]
Pink or humpback salmon (*Oncorhynchus gorbuscha*)	Liver	3.0	30,000[a]
Dover or slime sole (*Microstomus pacificus*)	Liver	7–19	5,000–31,000
	Viscera	0.5–1.5	1,000–30,00
Shad (*Alosa sapidissima*)	Liver	7.50	33,500[a]
	Viscera	9.00	15,000[a]
Dogfish or grayfish (*Squalus suckleyi*)	Liver	61–81	5,000–34,000
Columbia River chub (*Mylocheilus carinus*)	Liver	14.3	36,200[a]
	Viscera	39.5	3,750[a]
Turbot (*Pleuronichthys decurrens*)	Liver	16–20	16,000–37,000
Chinook salmon (*Oncorhynchus tshawytscha*)	Liver	8–10	25,000–40,000[a]
	Viscera	6.5	21,000[a]
School shark (*Galeorhinus australis*)	Liver	—	42,000–50,000
Albacore (*Germo germo*)	Liver	—	68,000
Jap or rough sole (*Lyopsetta exilis*)	Liver	5.55	71,800
Blenny eel (*Stichaedae* sp.)	Liver	9.67	76,200
Columbia River smelt (*Thaleichthys pacificus*)	Liver	12.2	32,600[a]
	Viscera	5.3	81,000[a]
Hagfish (*Polistotrema stoutii*)	Liver	4–24	2,000–102,000
Halibut (*Hippoglossus hippoglossus*)	Liver	—	110,000
Olive-backed rockfish (*Sebastodes saxicola*)	Liver	5–10	36,000–115,000
	Viscera	2–7	7,000–100,000
Tiger shark (*Galeocerdo tigrinus*)	Liver	7–75	20,000–137,000
Hammerhead shark (*Zygaena* sp.)	Liver	2–61	2,000–152,000
Black-finned shark (*Carcharias melanopterus*)	Liver	0.2–60	2,000–173,000
Petrale sole (*Eopsetta jordani*)	Liver	13–22	22,000–173,000
Soup-fin shark (*Galeorhinus zyopterus*)	Liver	45–65	48,000–190,000
	Liver	51–70	10,000–68,000
Black cod or sablefish (*Anoploma fimbria*)	Liver	14–20	150,000–200,000

[a] These sources also contain some vitamin A_2.

TABLE IX—*Continued*

Common and scientific name	Source of oil	Oil content, %	Vitamin A, I.U./g. oil
Black rockfish (*Scorpaenidae* sp.)	Liver	9–24	10,000–230,000
	Viscera	9.35	103,000
Red rockfish (*Scorpaenidae* sp.)	Liver	10–16	13,000–237,000
Swordfish (*Xiphias gladius*)	Liver	—	250,000
Ishnagi (*Stereolepis ishnagi*)	Liver	—	300,000
Pacific mackerel (*Decapturus* sp.)	Liver	6.71	324,000
Pacific halibut (*Hippoglossus stenolepis*)	Liver	11.27	17,000–193,000
	Viscera	3.6	361,000
Stonebass (*Polyprion americanus*)	Liver	—	400,000
Ling cod (*Ophiodon elongatus*)	Liver	9–23	50,000–500,000
California jewfish (*Stercolepis gigas*)	Liver	—	600,000

b. Isolation

The technique used for the isolation of vitamin A does not differ appreciably from that used for the isolation of the provitamins A. Vitamin A is quite frequently found in liver oils in the form of its esters[80] and sometimes is found bound to a protein molecule.[78] It is therefore necessary to saponify the liver oils under specified conditions in order to isolate the vitamin in the pure state. Furthermore, it is advisable to use an oil of very high potency or to concentrate low-potency oils by molecular distillation.[81] The concentrate is then heated for about one-half hour at 60 to 70° with 3 to 4 volumes of 10 % alcoholic potassium hydroxide while passing a stream of pure nitrogen through the mixture. Finally, the mixture is cooled and diluted with about ten times its volume of deoxygenated water and extracted with reagent-grade ethyl ether. The ether extract is then washed once with 10 % salt solution, with water, and dried. The ether is then removed under reduced pressure and the vitamin A concentrate further purified either by low temperature fractionation from methanol[82, 83] or by molecular distillation and subsequent recrystallization at low temperatures from ethyl formate.[84] The first method yields a product in the form of yellow needles, m.p. 7.5 to 8°, and contains methanol of crystallization;[83] the second method produces a product in yellow prisms free from solvent, m.p. 63 to 64°.

c. Physical and Chemical Properties

The crystals of vitamin A_1 are isotropic and are fairly stable when heated at moderate temperatures in an inert atmosphere and in the absence of

[80] A. O. Tischer, *J. Biol. Chem.* **125,** 475 (1938).
[81] K. C. D. Hickman, *Chem. Revs.* **34,** 51 (1944).
[82] N. A. Milas, U. S. Pat. 2,173,629 (Sept. 19, 1939).
[83] H. N. Holmes and R. E. Corbet, *J. Am. Chem. Soc.* **59,** 2042 (1937).
[84] J. G. Baxter and C. D. Robeson, *J. Am. Chem. Soc.* **64,** 2411 (1942).

light. Vitamin A_1 is optically inactive. It is soluble in most organic solvents but fails to dissolve in water, although water-soluble derivatives have been prepared.[85] Vitamin A_1 is unstable in the presence of oxygen or air and is usually stabilized by the addition of small amounts of antioxidants such

TABLE X

PROPERTIES OF VITAMIN A_1 AND SOME OF ITS DERIVATIVES

Vitamin A_1 or derivative	Molecular weight	M.p. or $n^{20°}_D$	λ_{max}, mμ (ethanol)	$E^{1\%}_{1\text{ cm.}}$	Biopotency, I.U./g. (Obs. × 10^{-6})	Biopotency, I.U./g. (Calcd. × 10^{-6})	References
Alcohol	286.4	64°	324–325	1835	3.33	—	86–89
Esters							
Acetate	328.5	57–58°	326	1550	2.91	2.904	86–89
Butyrate	356.5	1.5986	325–328	1345	2.2	2.675	91, 92
Lauroate	468.7	1.5668	325–328	1035	1.7	2.035	91, 92
Palmitate	524.8	28–29	325–328	975 (940)	1.6 (2.3)	1.817	(90), 91, 92
Stearate	552.9	1.5548	325–328	940	1.5	1.725	91, 92
Oleate	550.9	1.5559	325–328	870	2.0	1.731	91, 92
Benzoate	390.5	1.6305	325–328	1240	1.8	2.445	91, 92
Succinate	654.9	77–78°	325–328	1480 (1240)	2.5 (3.14)	2.912	91, 92 (90)
β-Napthoate	440.6	74–75°	328	(1090)	2.1 (3.44)	2.165	(90), 91, 93, 94
Anthraquinone-β-carboxylate	520.6	122–123°	330	(1065)	1.8	1.832	(89), 92
p-Phenylazobenzoate	494.6	80°	330	1540	—	1.928	89, 92
Ethers							
Methyl	300.5	34–35°	326[a]	1660	3.5	3.170	95
Phenyl	362.5	90–92°	327	1460	ca. 0.1	2.623	91

[a] In isopropanol.

[85] N. A. Milas, U. S. Pat. 2,443,373 (June 15, 1948).

[85a] Although vitamin A_1 is now considered to have the all-*trans* configuration, most of the early work was done with mixtures of stereoisomers. However, it is quite possible that most of the derivatives which have sharp melting points have only one configuration, presumably the all-*trans* form.

[86] J. Boldingh, H. R. Cama, F. D. Collins, R. A. Morton, N. T. Gridgeman, O. Isler, M. Kofler, R. J. Taylor, A. S. Welland, and T. Bradbury, *Nature* **168**, 598 (1951).

[87] H. R. Cama, F. D. Collins, and R. A. Morton, *Biochem. J.* **50**, 48 (1951).

[88] F. P. Zscheile and R. L. Henry, *Anal. Chem.* **14**, 422 (1942).

[89] J. G. Baxter, *Fortschr. Chem. org. Naturstoffe* **9**, 41 (1952).

[90] J. G. Baxter and C. D. Robeson, *J. Am. Chem. Soc.* **64**, 2407 (1942).

[91] O. Isler, A. Ronco, W. Guex, N. C. Hindley, W. Huber, K. Dialer, and M. Kofler, *Helv. Chim. Acta* **32**, 489 (1949).

[92] O. Isler, *Chimia* (*Switz.*) **4**, 103 (1950).

[93] S. Hamano, *Sci. Papers Inst. Phys. Chem. Research* (*Tokyo*) **28**, 69 (1935).

[94] T. H. Mead, *Biochem. J.* **33**, 589 (1939).

[95] A. R. Hanze, T. W. Conger, E. C. Wise, and D. I. Weisblat, *J. Am. Chem. Soc.* **70**, 1253 (1948).

as hydroquinone or α-tocopherol. The latter seems to be preferred, since it is known to have, in addition to its antioxygenic effect, a synergistic action on vitamins A.

Since vitamin A_1[85a] is an alcohol, it forms esters and ethers, most of which are more stable than the vitamin itself. The acetate has been adopted by the World Health Organisation as the international standard.[86, 87] The physi-

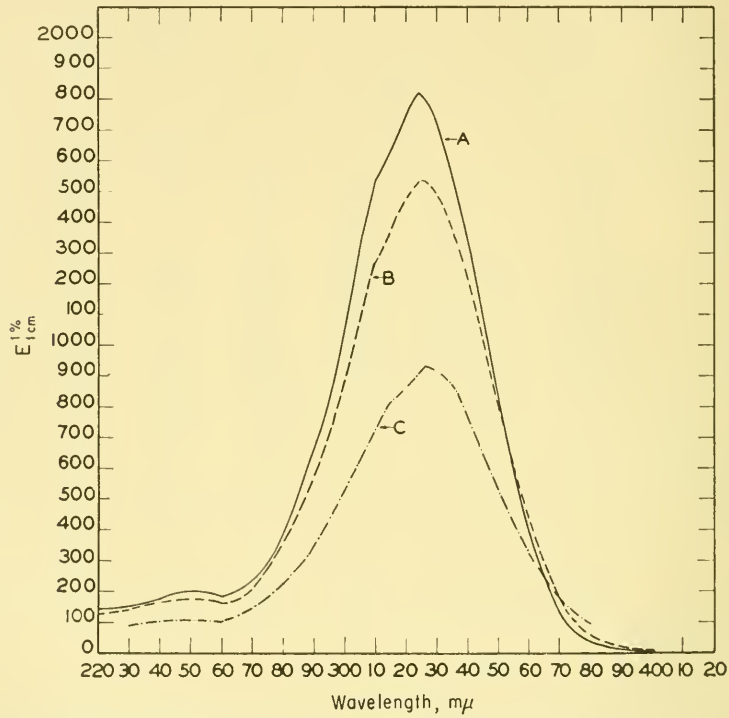


FIG. 4. Ultraviolet absorption spectra: A, all-*trans* vitamin A_1; B, all-*trans* vitamin A_1 acetate plotted from the data of Cama *et al.*;[87] and C, vitamin A_1 palmitate.[88]

cal properties of some of these derivatives together with their spectroscopic data and biopotencies are listed in Table X. Similar data for the all-*trans* vitamin A_1 are also included for comparison. The calculated biopotencies are based on the biopotencies of the all-*trans* vitamin A_1 and that of the all-*trans* vitamin A_1 acetate.[87] Figure 4 shows the ultraviolet absorption curves of the all-*trans* vitamin A_1 (curve A), the all-*trans* vitamin A_1 acetate (curve B), plotted from data published by Cama *et al.*,[87] and vitamin A_1 palmitate (curve C).[88] The configuration of vitamin A_1 palmitate was not definitely known when the measurements were made for this curve.

Vitamin A_1 is also known to react with antimony trichloride in chloroform

to give a deep blue color[96] which absorbs in the visible region of the spectrum with a characteristic and well-defined maximum at 620 mμ. The color is not stable, and after a few minutes it fades out with the appearance of a new maximum at 580 mμ. The maximum at 620 mμ has been used for a long time to measure the activity of vitamin A in liver oils and other vitamin A

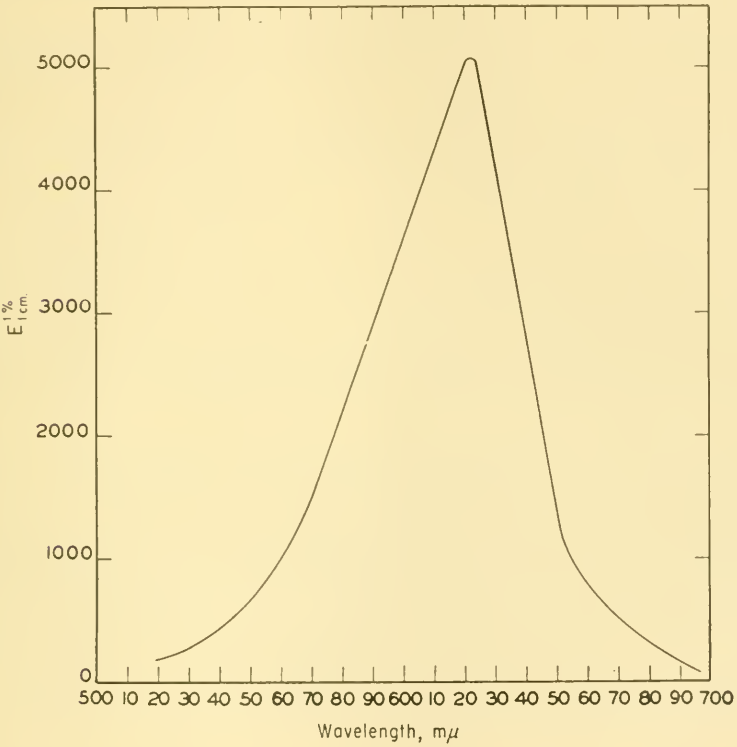


FIG. 5. The absorption spectrum of the reaction product of the all-*trans* vitamin A_1 with antimony trichloride in chloroform (cf. refs. 87 and 92).

preparations. An absorption curve of this color obtained with pure vitamin A_1 is shown in Fig. 5. The maximum $E^{1\%}_{1\,cm.}$ (620 mμ) for the all-*trans* vitamin A_1 is 5070, and that for the all-*trans* vitamin A_1 acetate is 4420.[87]

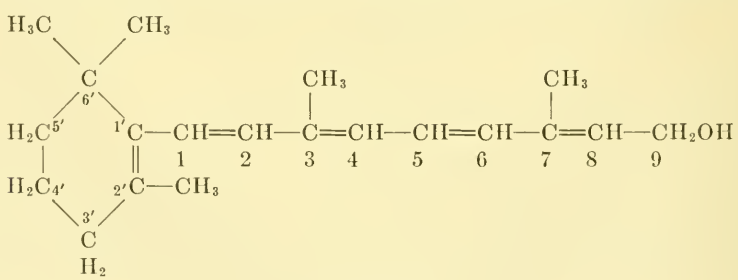
Vitamin A_1 reacts rapidly with maleic anhydride to form an adduct[97] which fails to give a blue color with antimony trichloride in chloroform. Mineral acids are known to destroy vitamin A_1 and its isomers, and, if this reaction is carried out with hydrogen chloride in ethanol, anhydrovitamin

[96] F. H. Carr and E. A. Price, *Biochem. J.* **20**, 498 (1926).
[97] K. Kawakami, *Sci. Papers Inst. Phys. Chem. Research (Tokyo)* **26**, 77 (1935).

A is formed which has very little or no biological activity. This reaction will be discussed more fully elsewhere.

d. Chemical Constitution

The structure of vitamin A was first elucidated by Karrer and his associates.[98] Notable contributions in this field were also made by Heilbron and his associates.[99] The empirical formula of vitamin A has been shown to be $C_{20}H_{30}O$. It has already been mentioned that vitamin A is an alcohol. That this alcohol is a primary one was shown by the careful oxidation of vitamin A to give an aldehyde (axerophthal).[100, 101] Upon catalytic hydrogenation vitamin A yields perhydrovitamin A with an empirical formula $C_{20}H_{40}O$. This indicates the presence in vitamin A of five double bonds and a carbon ring. That this ring is a six-membered ring and identical with that present in β-ionone or β-carotene was shown by ozonolysis which yielded 1 mole of geronic acid per mole of the vitamin. Careful oxidation with potassium permanganate in basic solution yielded 2 moles of acetic acid per mole of vitamin A, showing the presence of two methyl groups in the side chain. Similarly oxidation with hot chromic acid yielded 3 moles of acetic acid per mole of the vitamin, indicating the presence of three groups, $CH_3—\overset{|}{C}=$, in the molecule. Furthermore, selenium dehydrogenation yielded 2,6-dimethylnapthalene, which relates the structure of vitamin A to that of β-carotene (p. 9). Finally, Karrer and Morf[98b] synthesized perhydrovitamin A and showed it to be identical with that obtained by the complete hydrogenation of vitamin A. On the basis of these results Karrer proposed structure XLVI for vitamin A. This structure is in accordance with all the physical and chemical properties of vitamin A and



XLVI Vitamin A

[98] (a) P. Karrer, R. Morf, and K. Schöpp, *Helv. Chim. Acta* **14**, 1036, 1431 (1931). (b) P. Karrer and R. Morf, *Helv. Chim. Acta* **16**, 557, 625 (1933).

[99] I. M. Heilbron, R. N. Heslop, R. A. Morton, E. T. Webster, J. L. Rea, and J. C. Drummond, *Biochem. J.* **26**, 1178 (1932).

[100] R. F. Hunter and E. G. E. Hawkins, *J. Chem. Soc.* **1944**, 411.

[101] S. Ball, T. W. Goodwin, and R. A. Morton, *Biochem. J.* **42**, 516 (1948).

accounts for its formation from β-carotene (p. 24). That such a structure is capable of existence in *cis* and *trans* isomeric forms will be discussed elsewhere.

e. Synthesis

The synthesis of vitamin A has been the subject of numerous publications and patents, and the importance of this field may be judged by the appearance of several reviews in the short span of five years. This field has been reviewed by Embree,[102] Milas,[103] Johnson,[104] Heilbron,[105] Karrer,[106] Knobloch,[74] Inhoffen and Bohlmann,[107] Hunter,[108] Isler,[92] and Baxter.[89] It will be beyond the scope of this section to give a complete review of this field. However, an attempt will be made to give an accurate account of the important developments which led to our present knowledge of the synthesis of vitamin A.

There are three important routes to the synthesis of vitamin A, each of which is based on the application of some well-known reactions or key intermediates or both. One of these routes is based on the Reformatsky reaction or the addition of ethoxyacetylene to β-ionone. Another is based on the Darzens reaction for the synthesis of the key intermediate, C_{14}-aldehyde. A third synthesis is based on the use of 2,6,6-trimethylcyclohexanone as the key intermediate. It is now well recognized,[109] however, that some of the synthetic routes (Fig. 6 and part of Fig. 8) which lead to intermediates containing hydroxyl groups in direct allylic relationship to the double bond in the cyclohexyl ring are not suitable for the preparation of vitamin A in good yields. Although vitamin A has been prepared by such methods, the over-all yields were low and purification could be achieved only by elaborate methods to remove the undesirable isomeric intermediates.

(1) *Synthesis via the Reformatsky and Ethoxyacetylene Reactions.* The first attempt to synthesize vitamin A via the Reformatsky reaction was made by Kuhn and Morris,[110] but owing to a failure to separate the undesirable intermediates the yield of vitamin A in the final product was very small, and for the same reason subsequent investigators were unable to repeat the synthesis.[103] As our knowledge of the structure of the intermediates

[102] N. D. Embree, *Ann. Rev. Biochem.* **XVI**, 323 (1947).
[103] N. A. Milas, *Vitamins and Hormones* **5**, 1 (1947).
[104] A. W. Johnson, *Science Prog.* **36**, 496 (1948).
[105] I. Heilbron, *J. Chem. Soc.* **1948**, 386.
[106] P. Karrer, *Österr. Chem. Ztg.* **49**, 215 (1948).
[107] H. H. Inhoffen and F. Bohlmann, *Fortschr. chem. Forsch.* **1**, 175 (1949).
[108] R. F. Hunter, *Research (London)* **3**, 453 (1950).
[109] H. O. Huisman, A. Smit, S. Vromer, and L. G. M. Fischer, *Rec. trav. chim.* **71**, 899 (1952).
[110] R. Kuhn and C. J. O. R. Morris, *Ber.* **70**, 853 (1937).

became clarified,[89, 111, 112] it was recognized that in the dehydration of the hydroxy ester XLIX[113] large quantities of the undesirable β,γ-isomeric ester were produced in which the entire conjugated system shifted to the left of the terminal group so that the double bond in the ring was found to be in the same position as that present in the α-ionone ring. These undesirable intermediates led to a final preparation which had very little or no biological activity.

The discovery of the selective reduction properties of lithium aluminum hydride[114] and its immediate application to the synthesis of the C_{18}-ketone[103, 115] (XLVIII through LIV) led to a renewed interest in the synthesis of vitamin A via this route. The synthesis was completed by Schwarzkopf *et al.*,[116] by Wendler *et al.*,[117] the latter going through the C_{15}-aldehyde, LIII, and by Cawley *et al.*[118] The last group of investigators[119] claim the separation of the undesirable isomers from the dehydration products of both XLVIII and LV and their catalytic conversion, in the presence of catalysts such as phosphorus oxychloride, to the desirable isomers.

The C_{18}-ketone was first prepared[120, 121] by the Reformatsky reaction on β-ionone using ethyl-γ-bromocrotonate to form ethyl-β-ionylidenecrotonate, LX, which was hydrolyzed to the corresponding acid, LXI. When this acid was treated with methyl lithium a high yield of the C_{18}-ketone was obtained. This was converted by a second Reformatsky to the ester LVI, which was hydrolyzed to vitamin A acid.[121a] The vitamin A acid was also synthesized by a slightly different route.[122] These syntheses suffer the same limitations as the previous ones in that the undesirable isomers produced reduce considerably the over-all yield of the vitamin. In view of the crystal-

[111] H. Sobotka, E. Bloch, and D. Glick, *J. Am. Chem. Soc.* **65**, 1961 (1943).
[112] W. G. Young, L. J. Andrews, and S. J. Cristol, *J. Am. Chem. Soc.* **66**, 520 (1944).
[113] P. Karrer, H. Solomon, R. Morf, and O. Walker, *Helv. Chim. Acta* **15**, 878 (1932).
[114] A. E. Finholt, A. C. Bond, and H. I. Schlesinger, *J. Am. Chem. Soc.* **69**, 1199 (1947).
[115] N. A. Milas and T. M. Harrington, *J. Am. Chem. Soc.* **69**, 2247 (1947); **70**, 4275 (1948).
[116] O. Schwarzkopf, H. J. Cahnmann, A. D. Lewis, J. Swidinsky, and H. M. Wuest, *Helv. Chim. Acta* **32**, 443 (1949).
[117] N. L. Wendler, H. L. Slates, N. R. Trenner, and M. Tishler, *J. Am. Chem. Soc.* **71**, 3267 (1949); **73**, 719 (1951).
[118] J. D. Cawley, C. D. Robeson, E. M. Shantz, L. Weisler, and J. G. Baxter, U. S. Pat. 2,576,103 (Nov. 27, 1951).
[119] E. M. Shantz, C. D. Robeson, and H. M. Kascher, U. S. Pat. 2,576,104 (Nov. 27, 1951).
[120] J. F. Arens and D. A. van Dorp, *Nature* **157**, 190 (1926); *Rec. trav. chim.* **66**, 759 (1947); see also ref. 116.
[121] D. A. van Dorp and J. F. Arens, *Nature* **160**, 189 (1947).
[121a] D. A. van Dorp and J. F. Arens, *Rec. trav. chim.* **65**, 338 (1946).
[122] N. A. Milas, U. S. Pat. 2,424,994 (Aug. 5, 1947).

line nature and biological activity of the vitamin A acid, several investigators, in addition to those already mentioned, studied its preparation.[123-126]

The use of the Grignard of the ethoxyacetylene to prepare LI and, from it, the C_{15}-aldehyde, LIII, was also studied by several investigators.[127-129] A similar condensation was repeated with the C_{18}-ketone to give LVII, from which the vitamin A aldehyde, LIX, was prepared as outlined in Fig. 6. In these reactions some of the intermediates formed contain hydroxyl groups in direct allylic relationship to the double bond in the ring and are therefore subject to the limitations mentioned above.

(2) *Synthesis via C_{14}-Aldehyde.* The synthesis of vitamin A via C_{14}-aldehyde (Fig. 7) is not subject to the limitations imposed on the syntheses described in the previous section, and for this reason it is the preferred synthesis. C_{14}-Aldehyde was first synthesized by Ishikawa and Matsuura[130] by the application of the Darzens reaction to β-ionone. The application of this aldehyde to the synthesis of vitamin A was first recognized by Milas[131a] and later by Heilbron *et al.*[132] and by Isler *et al.*[133a] Since the C_{14}-aldehyde may exist in more than one form, its structure has been the subject of several investigations.[134-139] If the aldehyde is purified by chromatography with activated alumina, the product obtained is the same irrespec-

[123] I. Heilbron, E. R. H. Jones, and D. G. O'Sullivan, *J. Chem. Soc.* **1946**, 866.
[124] P. Karrer, E. Jucker, and E. Schick, *Helv. Chim. Acta* **29**, 704 (1946).
[125] H. H. Inhoffen, F. Bohlmann, and M. Bohlmann, *Ann.* **568**, 47 (1950).
[126] H. O. Huisman, *Rec. trav. chim.* **69**, 851 (1950).
[127] J. F. Arens, D. A. van Dorp, G. van Dijk, and B. J. Brandt, *Rec. trav. chim.* **67**, 973 (1948).
[128] N. A. Preobrazhenskiĭ and I. A. Rubstov, *J. Gen. Chem.* (*U. S. S. R.*) **18**, 1719 (1948).
[129] I. Heilbron, E. R. H. Jones, and B. C. L. Weedon, *J. Chem. Soc.* **1949**, 1823.
[130] S. Ishikawa and T. Matsuura, *Sci. Repts. Tokyo Bunrika Daigaka* **3A**, 173 (1937).
[131] (a) N. A. Milas, U. S. Pat. 2,369,156 (filed Aug. 22, 1940; issued Feb. 13, 1945, and several additional patents issued in 1945; see ref. 103).
(b) *Science* **103**, 581 (1946).
[132] I. Heilbron, A. W. Johnson, E. R. H. Jones, and A. Spinks, *J. Chem. Soc.* **1942**, 727.
[133] (a) O. Isler, M. Koefler, W. Huber, and A. Ronco, *Experientia* **2**, 31 (1946).
(b) *Helv. Chim. Acta* **30**, 1911 (1947).
[134] J. Cymerman, I. Heilbron, E. R. H. Jones, and R. N. Lacey, *J. Chem. Soc.* **1946**, 500.
[135] N. A. Milas, S. W. Lee, E. Sakal, H. C. Wohlers, N. S. MacDonald, F. X. Grossi, and H. F. Wright, *J. Am. Chem. Soc.* **70**, 1584 (1948).
[136] G. W. H. Cheeseman, I. Heilbron, E. R. H. Jones, F. Sondheimer, and B. C. L. Weedon, *J. Chem. Soc.* **1949**, 1516.
[137] H. H. Inhoffen, F. Bohlmann, and G. Linhoff, *Ann.* **570**, 73 (1950).
[138] Y-R. Naves, H. Barbier, and P. Ardizio, *Bull. soc. chim. France* **1950**, 1188.
[139] N. A. Milas, P. Davis, and M. T. Burgess, Unpublished results.

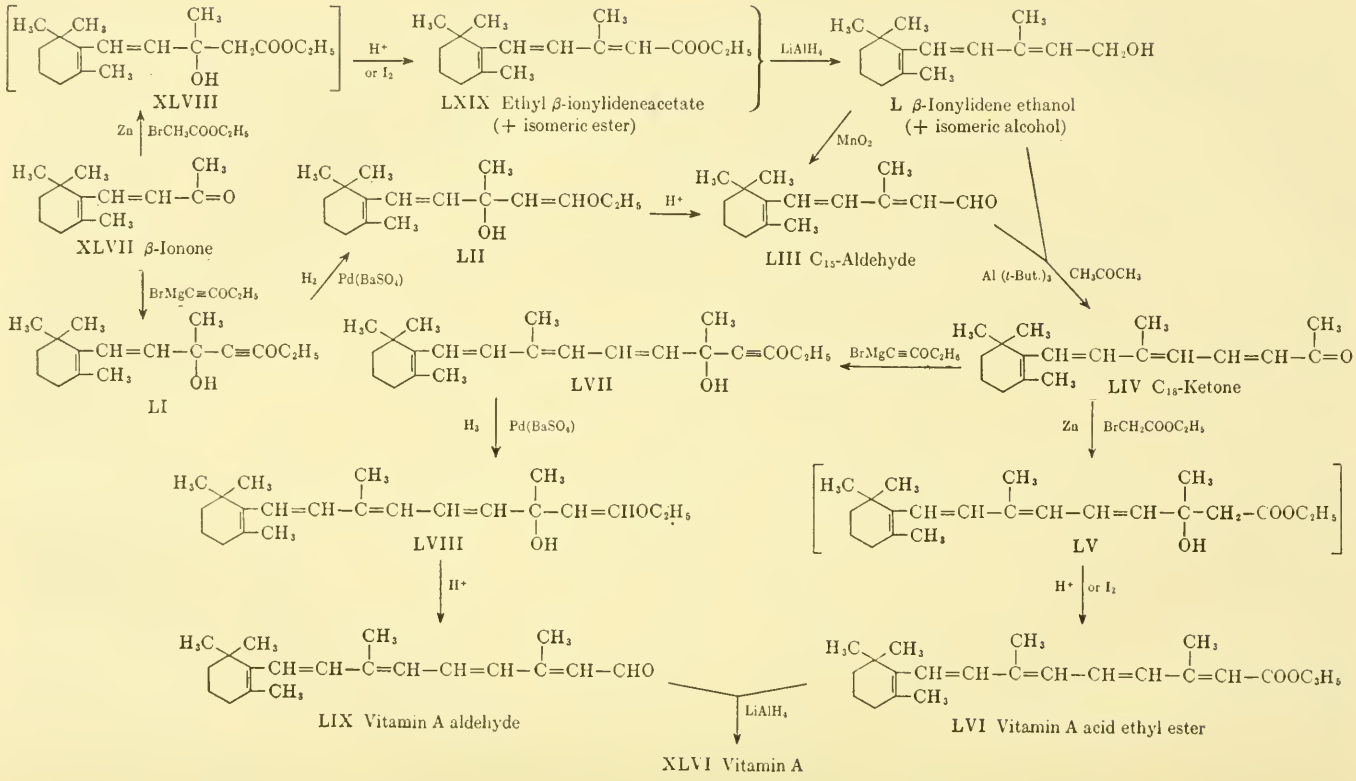


FIG. 6. Synthesis of vitamin A via the Reformatsky and ethoxyacetylene reactions.

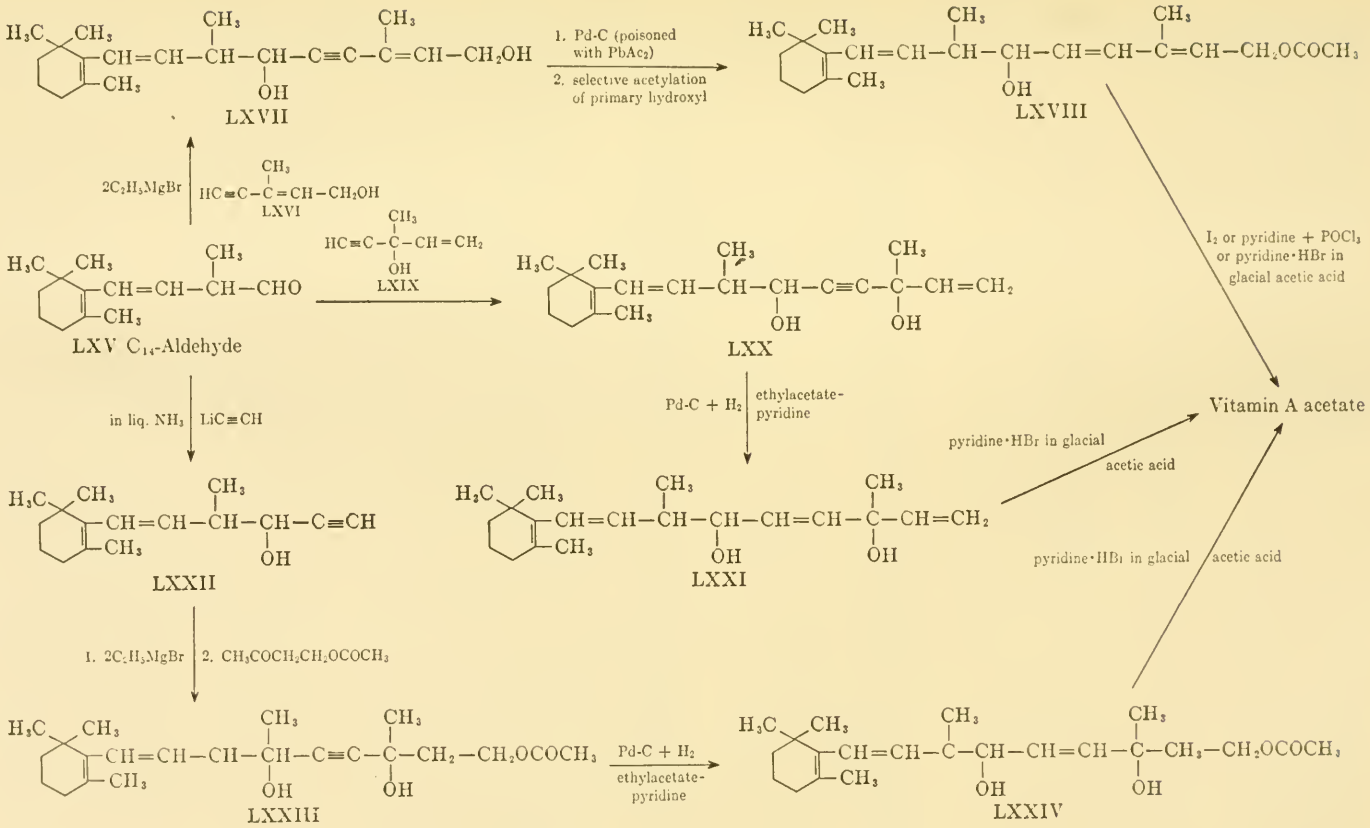
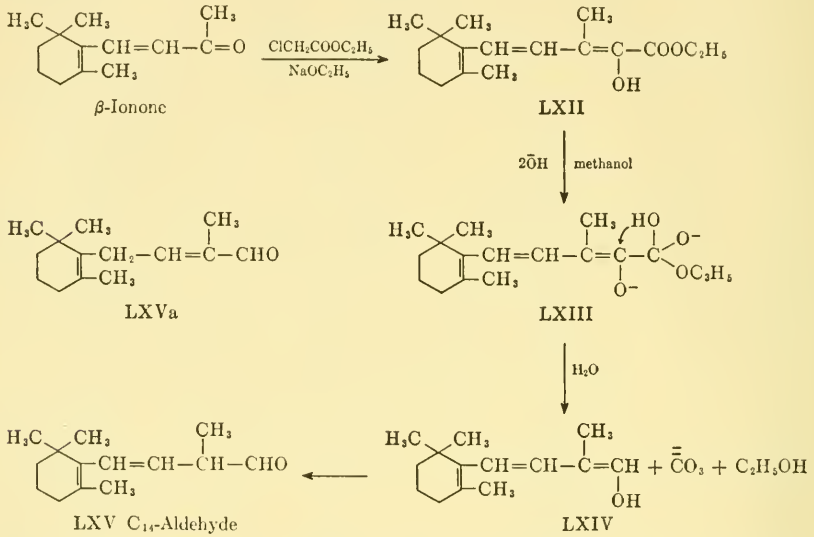


FIG. 7. Synthesis of vitamin A via C_{14}-aldehyde.

tive of the variations in the method of preparation.[139] The chemical evidence accumulated in the author's laboratory during the past several years is still strongly in favor of structure LXV, which will be used throughout the present report. An improved method for the preparation of C_{14}-aldehyde has been patented by Lindlar.[140] The various intermediates involved in this preparation are best expressed by the following equations which are based on the most recent extended study of this reaction.[139]



Referring to Fig. 7, the first practical method[131] for the synthesis of vitamin A involves the condensation via the Grignard reaction of C_{14}-aldehyde with the acetylenic carbinol LXIX to form the glycol LXX. This is selectively hydrogenated in a 50–50 ethyl acetate–pyridine mixture using palladium deposited on charcoal to give the glycol LXXI which undergoes dehydration accompanied by allylic rearrangement and acetylation in glacial acetic acid and in the presence of pyridine hydrobromide.[141-143] The yields of vitamin A acetate are equally as high as those obtained by the sequence of reactions using the Grignard of the acetylenic carbinol LXVI.[91, 133b, 144-146] In this case the important step is that which makes use of the poisoned palladium catalyst.[147] Partial hydrogenation of the acetylene bond

[140] H. Lindlar, U. S. Pat. 2,451,740 (Oct. 19, 1948); see also ref. 133b.
[141] N. A. Milas, U. S. Pat. 2,577,538 (Dec. 4, 1951).
[142] N. A. Milas, British Pat. 664,815 (Jan. 16, 1952).
[143] N. A. Milas, Swiss Pat. 288,645 (March 11, 1953).
[144] O. Isler, U. S. Pat. 2,451,739 (Oct. 19, 1948).
[145] N. A. Milas, U. S. Pat. 2,567,572 (Sept. 11, 1951).
[146] O. Isler, *Chem. Eng. News* **29**, 3962 (1951).
[147] H. Lindlar, *Helv. Chim. Acta* **35**, 446 (1952).

seems to be entirely selective, and the desired glycol is obtained in high yields. Although in the original process selective acetylation of the primary hydroxyl group is essential prior to the final step, it can be omitted if the reaction is carried out in glacial acetic acid using pyridine hydrobromide as the dehydrating agent.

Vitamin A acetate was also prepared by a slightly different route.[131b, 148] The acetylenic carbinol LXXII was condensed via the Grignard reaction with 4-acetoxybutanone-2 to yield the 5-dehydroglycol acetate LXXIII which was selectively hydrogenated to the glycol LXXIV. Upon dehydration of this glycol in glacial acetic acid, using pyridine hydrobromide, vitamin A acetate was obtained in good yields. These reactions have been repeated recently,[149] and the over-all yield of vitamin A acetate obtained was somewhat lower than that obtained in the other two methods shown in Fig. 7.

Synthetic purified vitamin A acetate and vitamin A obtained from it by hydrolysis were found to be identical in every respect with the natural vitamin A and its acetate.

Vitamin A ethers have also been synthesized in good yields by methods[133a, 150, 151] analogous to those outlined in Fig. 7. Other methods,[152, 153] which are subject to the limitations mentioned on p. 34, produced very little or no biologically active ethers.

(3) *Synthesis via 2,6,6-Trimethylcyclohexanone.* The synthesis of vitamin A via this route is outlined in Fig. 8.[154] 2,6,6-Trimethyl-1-ethynylcyclohexanol-1, LXXVI, was prepared[155, 156] in good yields by the addition of sodium acetylide in liquid ammonia to trimethylcyclohexanone, LXXV. This was condensed via the Grignard reaction with the ketone LXXVII[157] to yield the glycol LXXVIII, which was allowed in a homogeneous acidic medium to undergo an anionic rearrangement to give the glycol LXXIX. It was found impossible to reduce this glycol or the glycol LXXXIII selectively by any ordinary means of reduction except by the use of lithium aluminum hydride previously applied to this type of compounds by Sobotka

[148] N. A. Milas, U. S. Pat. 2,369,168 (Feb. 13, 1945).

[149] N. A. Milas, P. Davis, and M. T. Burgess, Unpublished results.

[150] N. A. Milas, U. S. Pat. 2,369,157 (Feb. 13, 1945).

[151] N. A. Milas, E. Sakal, J. T. Plati, J. T. Rivers, J. K. Gladding, F. X. Grossi, Z. Weiss, M. A. Campbell, and H. F. Wright, *J. Am. Chem. Soc.* **70**, 1597 (1948).

[152] F. B. Kipping and F. Wild, *Chemistry & Industry* **1939**, 802.

[153] W. Oroshnik, G. Karmas, and A. D. Mebane, *J. Am. Chem. Soc.* **74**, 295 (1952).

[154] J. Attenburrow, A. F. B. Cameron, J. H. Chapman, R. M. Evans, B. A. Hems, A. B. A. Jansen, and T. Walker, *J. Chem. Soc.* **1952**, 1094.

[155] N. A. Milas, N. S. MacDonald, and D. B. Black, *J. Am. Chem. Soc.* **70**, 1829 (1948).

[156] H. Sobotka and J. D. Chanley, *J. Am. Chem. Soc.* **71**, 4136 (1949).

[157] G. W. H. Cheeseman, I. Heilbron, E. R. H. Jones, F. Sondheimer, and B. C. L., Weedon, *J. Chem. Soc.* **1949**, 2031.

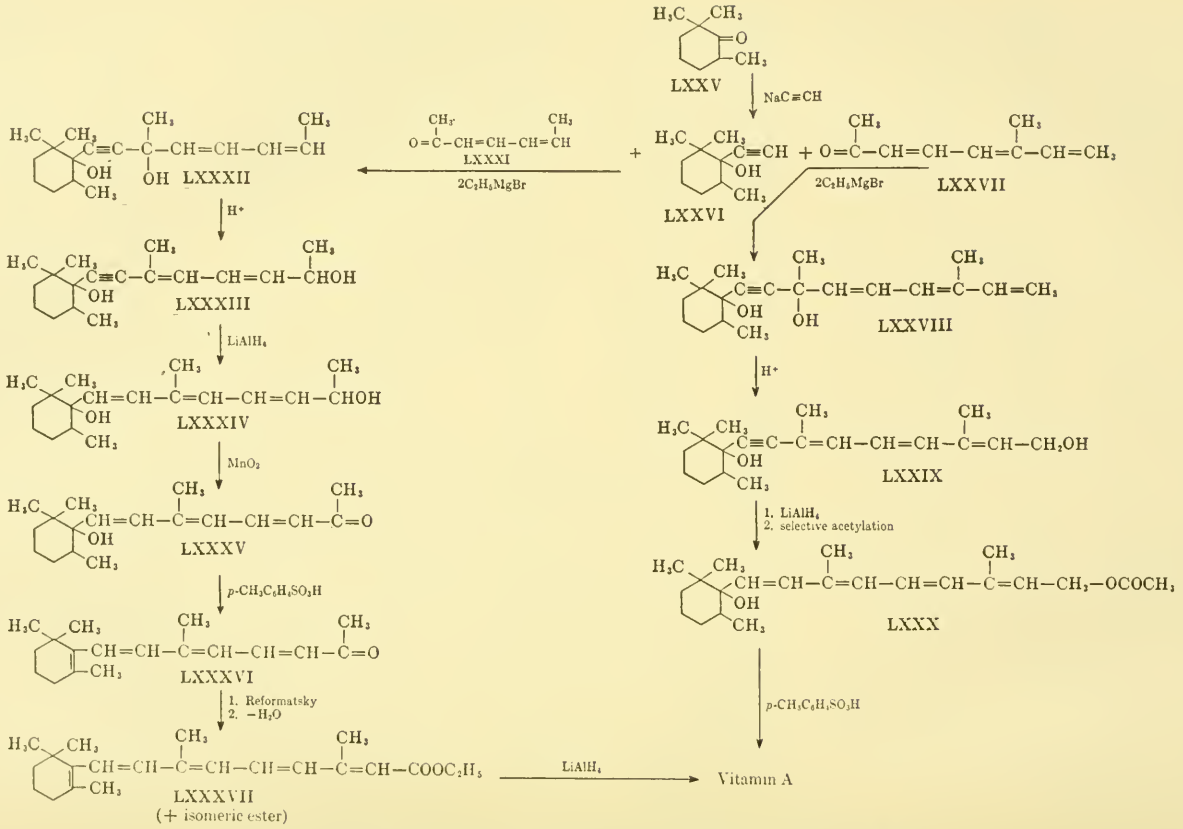


FIG. 8. Synthesis of vitamin A via 2,6,6-trimethylcyclohexanone.

and Chanley.[158] The reduced glycol was then selectively acetylated, and the monoacetate LXXX was dehydrated with *p*-toluenesulfonic acid in boiling benzene or toluene. A mixture of about 50 % of vitamin A acetate, anhydrovitamin A, and unchanged starting material was obtained. Further purification was effected by chromatography and by converting the final product into crystalline vitamin A anthraquinone-2-carboxylate, the identity of which was confirmed by comparison with an authentic sample, ultraviolet absorption spectra, and biological tests. Although this process is of theoretical interest, it does not afford an advantage over the processes outlined in Fig. 7. The preparation of the key intermediates LXXVI and LXXVII involves several steps, some of which give low yields of the desired products, and the overall yields of the vitamin itself are low. Moreover, the use of the expensive reagent, $LiAlH_4$, should always be considered in any economical synthesis.

The alternative route by way of compound LXXXII and the C_{18}-ketone LXXXVI is less satisfactory, since it is subject to the same limitations inherent in the methods outlined in Fig. 6.

f. Industrial Preparation

For a long time vitamin A and vitamin A products have been of considerable commercial importance, and in 1950 the demand for vitamin A in the United States alone was estimated at 120 trillion U.S.P. units.[159] One of the oldest methods of concentrating the vitamin from fish liver oils has already been described elsewhere. With the advent of commercial methods of molecular or high-vacuum distillations low-potency oils were concentrated successfully by this method without much loss of potency.[81] Prior to the development of economical synthetic methods most of the high-potency concentrates, in the form of esters or mixtures of esters and the vitamin, were produced industrially by this method.

Of all the synthetic processes the most economical one and the one preferred and widely practiced in the United States by Hoffmann-La Roche, Pfizer, and Merck, and by Hoffman-La Roche in Europe is that which uses C_{14}-aldehyde as the key intermediate. The first two methods outlined in Fig. 7 give approximately the same over-all yields of vitamin A. However, the first process was originally adopted industrially by Hoffmann-La Roche[144, 146] and is essentially the one used by the others. In some of the formulations of this process the structure LXVa of the C_{14}-aldehyde is used, although from the practical point of view it is immaterial, since the aldehyde used by all the investigators in this field was essentially the same.

The starting materials for this process include citral, the main constituent

[158] J. D. Chanley and H. Sobotka, *J. Am. Chem. Soc.* **71**, 4140 (1949).
[159] Staff Report, *Ind. Eng. News* **29**, 1316 (1951).

of lemon grass oil, acetone, and formaldehyde. Citral is first condensed by an old and well-known process with acetone to form pseudoionone which is cyclized, in the presence of mineral acids, to β-ionone which has been an article of commerce for many decades. β-Ionone is then converted via the Darzens reaction (p. 35) to give an over 80 % yield of C_{14}-aldehyde. To prepare the other key intermediate, LXVI, acetone is first condensed with formaldehyde in weak basic solution, and the product formed is dehydrated to give methyl vinyl ketone. This ketone has also been produced industrially by the alcoholysis of vinyl acetylene. Methyl vinyl ketone is then allowed to react with lithium acetylide in liquid ammonia under a slightly increased pressure of acetylene to give the acetylenic carbinol LXIX in good yields. The hazardous nature of acetylene under pressure requires concave construction of the equipment to resist blasts in the event of an explosion. The acetylenic carbinol is then allowed to undergo an anionic rearrangement, in the presence of dilute mineral acids, to give the acetylenic carbinol LXVI. When this carbinol is allowed to react via the Grignard reaction with C_{14}-aldehyde, the solid C_{20}-acetylenic glycol LXVII (m.p. 59°) is produced in yields of 90 % or over. This glycol is selectively hydrogenated using the poisoned palladium catalyst mentioned elsewhere to give almost quantitative yields of the olefinic glycol which is acetylated at 0° with one equivalent of acetic anhydride in the presence of pyridine to give the C_{20}-glycol monoacetate LXVIII (m.p. 74°). Several methods have been employed to carry out the final dehydration step. In the original announcement[144] iodine was used to carry out the dehydration of the C_{20}-glycol monoacetate to give vitamin A acetate, but later[146] phosphorus oxychloride in pyridine was found to give better results. Yields up to 45 % were reported in this step. The yields were increased by recovering the unconverted C_{20}-glycol acetate and retreating it in the same manner. To protect the vitamin A acetate during its formation it is necessary to use small amounts of α-tocopherol together with the C_{20}-glycol monoacetate. With pyridine hydrobromide or pyridine *p*-toluenesulfonate in pyridine or in glacial acetic acid, yields as high as 70 % of the vitamin A acetate have been reported.[141] The over-all yields of vitamin A from β-ionone are about 25 to 45 %.

The synthetic vitamin A is marketed either as the acetate or the palmitate. Pfizer has developed a crystalline preparation of either the acetate or the palmitate coated with gelatin. This preparation is tasteless and odorless and maintains its potency in air at 45° for over 1000 hours.[159] It is also claimed that this preparation is more easily utilized by human beings than other preparations, and when fed to chickens or hogs in conjunction with terramycin and penicillin it is absorbed more efficiently than when fed alone.[160] The industrial preparations of synthetic vitamin A usually range

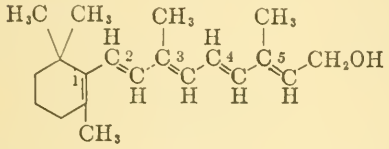
[160] H. G. Luther, *Ind. Eng. News* **30**, 539 (1952).

in potency from 500,000 to 1,600,000 U.S.P. units per gram and are sold at this writing at a price of 12 cents per million units.

3. Stereoisomers of Vitamin A

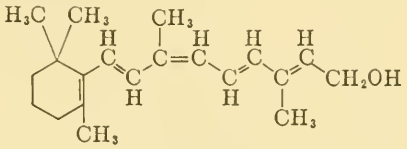
a. Stereoisomeric Considerations

It has already been mentioned elsewhere that vitamin A (structure XLVI) may exist in several stereoisomeric forms. Of the five double bonds present, only four in the side chain can contribute toward stereoisomerism, and owing to the existence of steric hindrance[37-39] only two (3 and 5) may assume the *cis* configuration. On this basis there should be four stereoiso-



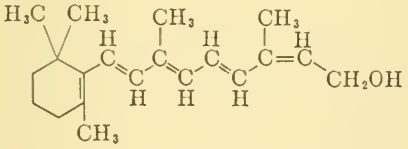
LXXXVIII

2,3,4,5-Tetra-*trans* vitamin A or vitamin A_1



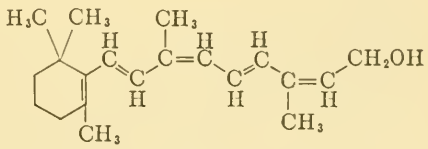
LXXXIX

2,4,5-Tri-*trans* 3-*cis* vitamin A



XC

2,3,4-Tri-*trans* 5-*cis* vitamin A or neovitamin A



XCI

2,3-Di-*trans* 3,5-di-*cis* vitamin A

Fig. 9. Stereoisomers of vitamin A.

mers of vitamin A as shown in Fig. 9. Of these only two are definitely known; the all-*trans* form, commonly known as vitamin A_1, and neovitamin A which has probably the 5-*cis* configuration. It is quite possible that under certain conditions all stereoisomers may exist in nature at the same time, and, like their precursors, they can be interconverted on exposure to light in the presence of traces of iodine.[161]

b. Neovitamin A or 2,3,4-Tri-trans 5-cis Vitamin A

This vitamin was recognized by Robeson and Baxter,[162] who isolated it from the "non-crystallizable portion" of natural vitamin A samples. It was found to constitute about 35% of the total vitamin A present in fish liver

[161] R. Hubbard and G. Wald, *Science* **115**, 60 (1952).

[162] C. D. Robeson and J. G. Baxter, *J. Am. Chem. Soc.* **69**, 136 (1947).

oils. Even samples of synthetic vitamin A contain neovitamin A.[163] Table XI shows the occurrence of neovitamin A in some fish liver oils, concentrates, distillates, and synthetic vitamin A.

The neovitamin A was purified by Robeson and Baxter[162] via its *p*-phenylazobenzoate (m.p. 94 to 96°) which was saponified and the vitamin regenerated and crystallized from ethyl formate at −70° (m.p. 58 to 60°). The neovitamin A anthraquinone β-carboxylate was also prepared (m.p. 134 to 136°) and found to be red as contrasted with the corresponding ester of the all-*trans* isomer, which is yellow. Neovitamin A has a well-defined maximum in the ultraviolet at 328 mμ, with an $E^{1\%}_{1\text{ cm.}}$ value in isopropanol

TABLE XI

Occurrence of Neovitamin A in Fish Liver Oils and Synthetic Vitamin A Preparations

Sample	Neovitamin A, in % of total vitamin A	References
U. S. Reference Cod Liver Oil No. 3	39	162
Dogfish liver oil	36	162
Soupfin shark liver oil	37	162
Halibut liver oil	32	162
California jewfish liver oil	34	162
Spanish red tuna liver oil	55	164
Distillate from dogfish liver oil	39	162
Distillate from soupfin shark liver oil	33	162
Unsaponifiable matter from soupfin shark distillate	34	162
Distilled vitamin A concentrates	39, 35	162
Synthetic vitamin A	40, 34	163

of 1673 (cf. ref. 165). The infrared spectra of the two stereoisomers are almost identical.

Chemically neovitamin A is more stable to air oxidation, slightly less strongly adsorbed on weakened alumina (5% water), and forms anhydrovitamin A more slowly than the all-*trans* isomer. Maleic anhydride reacts rapidly with the all-*trans* vitamin A to form an adduct which fails to give the Carr-Price test,[97] whereas neovitamin A reacts at a much slower rate. For example, after treatment of neovitamin A palmitate and vitamin A_1 palmitate with maleic anhydride in benzene for 16 hours, 90% of the former and only 5% of the latter were recovered unchanged as measured by the antimony trichloride color test. This method has been adopted for the assaying of neovitamin A in the presence of vitamin A_1.[162-165]

[163] J. D. Cawley, C. D. Robeson, L. Weisler, E. M. Shantz, N. D. Embree, and J. G. Baxter, *Science* **107**, 346 (1948).

[164] P. Meunier and J. Jouanneteau, *Bull. soc. chim. biol.* **30**, 260 (1948).

[165] P. D. Dalvi and R. A. Morton, *Biochem. J.* **50**, 43 (1952).

Recently Harris *et al.*[166] have determined the biopotency of neovitamin A alcohol and neovitamin A acetate. They found that the alcohol has a potency of 87.1 ± 3.25% of the potency of all-*trans* vitamin A and the acetate has a potency of 83.2 ± 4.28% of the potency of all-*trans* vitamin A acetate. Furthermore, the authors came to the conclusion that in a mixture of the two stereoisomers each isomer exerts its characteristic physiological effect independently of the presence of the other isomer. The same authors also studied the extent of interconversion of the two stereoisomers *in vivo*. They found that rats have the ability to convert neovitamin A to the all-*trans* form, and vice versa, and they seem to store in their livers a mixture of the two isomers containing approximately 12% of neovitamin A and 88% of the all-*trans* vitamin A, regardless of which pure isomer is fed. This ratio seems to be different with different species; in fish livers it is about 35:65.

Chemical interconversion of neovitamin A and vitamin A_1 has also been accomplished through their anthraquinone β-carboxylate esters by treatment with traces of iodine in benzene solution. The neovitamin A ester was converted to the corresponding vitamin A_1 ester to the extent of about 70% in 2 hours at 25°, whereas the reverse was accomplished under similar conditions to the extent of only 30%.[162]

From the chemical and biological behavior of neovitamin A, it was concluded that one of the double bonds must have the *cis* configuration, and, since neovitamin A dehydrates at a much slower rate than vitamin A_1, the *cis* double bond must be the one closer to the hydroxyl group. This reasoning is not entirely clear, since the dehydration can be almost completely prevented by interposing a triple bond between the hydroxyl group and the double bond in the ring. For example, 1,2-dehydrovitamin A, which forms both the red and the yellow anthraquinone β-carboxylate esters, is not easily dehydrated with methanolic hydrogen chloride.[154] Furthermore, the red color of the anthraquinone β-carboxylate and the position of the ultraviolet absorption maximum have not been adequately explained on the basis of the *cis* configuration. It is well known that the *cis* isomers in the carotenoid group (Section II, A) absorb at lower wavelengths than the corresponding all-*trans* isomers. Moreover, the 3-*cis* vitamin A aldehyde seems to follow this rule.[167]

c. Evidence for the Possible Existence of Other Stereoisomers

Mention has already been made of the 3-*cis* vitamin A aldehyde which can easily be converted to the 3-*cis* vitamin A. Hubbard and Wald have reported recently [161] that natural vitamin A concentrates can be converted *in vitro* to rhodopsin in the presence of opsin and two enzymes, liver alcohol

[166] P. L. Harris, S. R. Ames, and J. H. Brinkman, *J. Am. Chem. Soc.* **73**, 1252 (1951).
[167] W. Graham, D. A. van Dorp, and J. F. Arens, *Rec. trav. chim.* **68**, 609 (1949).

dehydrogenase and cozymase. It is surprising that when the natural vitamin A was replaced with crystalline vitamin A and neovitamin A the system failed to synthesize rhodopsin. However, when solutions of the crystalline vitamins were exposed to light in the presence of traces of iodine, the products formed became effective precursors of rhodopsin. Since enzymes are known to be stereospecific, it is quite possible that other stereoisomers than the all-*trans* vitamin A and neovitamin A are responsible for the production of rhodopsin. But both the all-*trans* vitamin A and neovitamin A are effective in all body processes including vision; therefore it is quite likely that they are isomerized *in vivo* to the stereospecific isomer which is responsible for the production of rhodopsin.

4. Vitamin A Aldehyde (Retinene)

Vitamin A aldehyde (LIX, Fig. 6) may be considered as an oxidation product of vitamin A. It was first isolated from retinas by Wald,[168] who named it retinene, and it was later recognized as the vitamin A aldehyde by Morton.[169] Its importance in the visual processes warrants here a brief description of its preparation and physical and chemical properties.

Vitamin A aldehyde was first prepared in small yields by the direct oxidation in dilute acid solution of vitamin A with potassium permanganate[170] and by the application of the Oppenauer reaction to vitamin A.[100] Better yields were obtained by the oxidation of vitamin A with a specially prepared manganese dioxide.[101] It has also been obtained by the oxidation of β-carotene.[4-7] A direct synthesis from C_{18}-ketone (LIV $\rightarrow$ LVII $\rightarrow$ LVIII $\rightarrow$ LIX, Fig. 6) was published by Arens and van Dorp.[171]

Vitamin A aldehyde, like vitamin A, should exist in four stereoisomeric forms.[172] The one described in the previous paragraph is presumably the all-*trans* form. The 3-*cis* isomer was synthesized via the 3-*cis* C_{18}-ketone according to the same sequence of reactions used for the synthesis of the all-*trans* form (Fig. 6).[167] Neovitamin A aldehyde or neoretinene (probably the 5-*cis* form) was prepared by the oxidation of neovitamin A with manganese dioxide.[161, 165] The physical properties and some of the derivatives of these aldehydes or retinenes are given in Table XII.

The 2,4-dinitrophenylhydrazones of retinene$_1$ and neoretinene were also prepared[101, 165] and were found to have essentially the same m.p. 207 to 208° (214 to 215°[173]). With antimony trichloride in chloroform retinene$_1$ gives a

[168] G. Wald, *J. Gen Physiol.* **19**, 351 (1935).
[169] R. A. Morton, *Nature* **153**, 69 (1944).
[170] R. A. Morton and T. W. Goodwin, *Nature* **153**, 405 (1944).
[171] J. F. Arens and D. A. van Dorp, *Rec. trav. chim.* **68**, 604 (1949).
[172] P. Meunier and J. Jouanneteau, *Bull. soc. chim. biol.* **30**, 260 (1948).
[173] K. R. Farrar, J. C. Hamlet, H. B. Henbest, and E. R. H. Jones, *J. Chem. Soc.* **1952**, 2657.

single maximum at 664 mμ, $E^{1\%}_{1\,cm.}$ 3400. Under similar conditions neoretinene gives a maximum at 662 mμ. The enzyme system of Hubbard and Wald failed to synthesize rhodopsin when either retinene$_1$ or neoretinene was added to it.[161] The biopotency of what may be the all-*trans* vitamin A aldehyde has been reported as essentially the same as that of natural crystalline vitamin A_1.[171] A more recent determination is given as 2.8×10^6

TABLE XII

Physical Properties of Vitamin A Aldehydes (Retinenes)

(Aldehyde Retinene)	M.p.	λ_{max}, mμ	$E^{1\%}_{1\,cm.}$	Solvent	References
2,3,4,5-Tetra-*trans*	—	368	1050	Ethanol	100
(Retinene$_1$)	61–62°	369.5	1685	Petroleum ether	101
		373	1548	Cyclohexane	101
	(64–65°)	385.5	1400	Ethanol	101 (173)
		389	1303	Chloroform	101
	61–64°	375	1090	Ethanol	171
	57°	381	1530	Ethanol	89
2,3,4,5-Tetra-*trans*	193–195°	385	2062	Chloroform	101
semicarbazone	161–164°	385	1742	Chloroform	101
	188–190°	385	1540	Chloroform	6
	199–201° (d.)	385	1860	Chloroform	6
	207–209° (d.)	375	2180	Chloroform	171
2,4,5-Tri-*trans* 3-*cis* (retinene?)	57.5–60°	372	930	Ethanol	167
2,4,5-Tri-*trans* 3-*cis* semicarbazone	193–195°	371	1610	Chloroform	167
2,3,4-Tri-*trans* 5-*cis*	—	380	—	Ethanol	165
(neoretinene)	—	377	—	Ethanol	161
2,3,4-Tri-*trans* 5-*cis* semicarbazone	185°	—	—	—	165

I.U. per gram.[6] The biopotency of the 3-*cis* isomer is also the same as that of vitamin A_1.[167]

5. Vitamin A Acid

Vitamin A acid has not been isolated from natural sources, although it could conceivably be formed from vitamin A. However, it has been synthesized by several investigators (see p. 34). The all-*trans* form is a crystalline product which has the properties shown in Table XIII. The spectroscopic data were determined in ethanol.

The all-*trans* vitamin A acid has a biopotency of about two-thirds that of crystalline vitamin A_1.

TABLE XIII

Physical Properties of Vitamin A Acid

M.p.	λ_{max}, mμ	$E\,\frac{1\%}{1\,cm.}$	References
181.5°	347	1460	121a
181.5°	353	1510	117
179–180°	347	1600	125
180°	350	1507	89

6. Anhydrovitamin A_1

When vitamin A in all its isomeric forms is treated with small concentrations of hydrogen chloride in ethanol, a hydrocarbon is formed whose ultra-

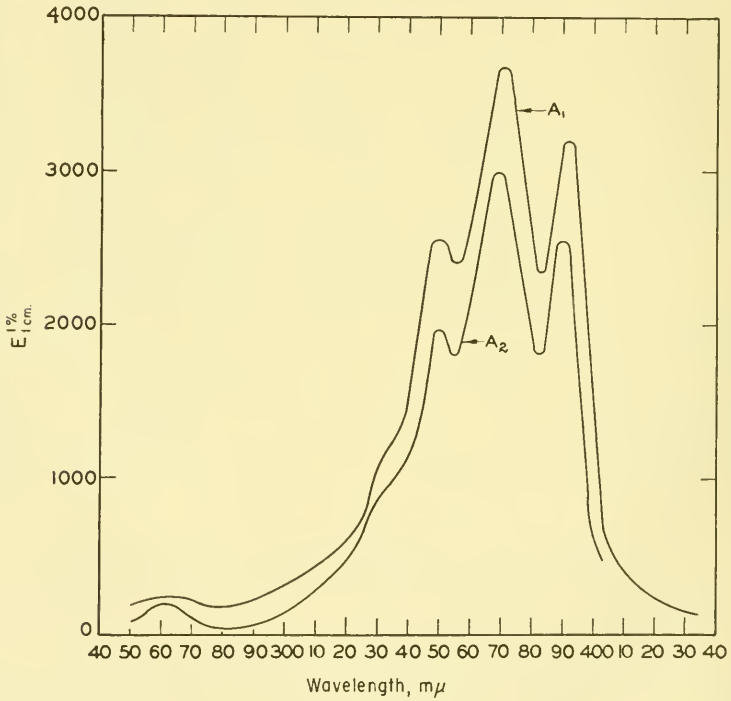
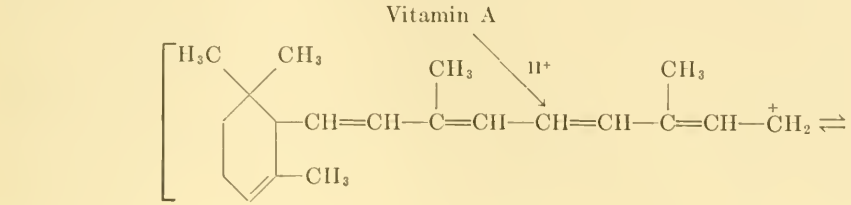


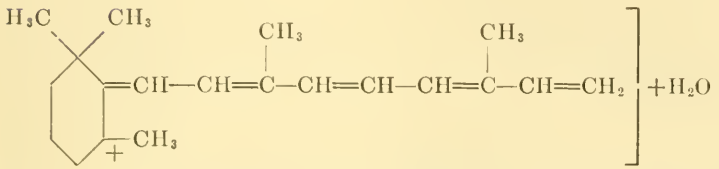
Fig. 10. Ultraviolet absorption spectra of anhydrovitamin A_1 and anhydrovitamin A_2.

violet absorption spectrum shows a fine structure of three maxima at 351, 371, and 392 mμ, respectively (Fig. 10). The same hydrocarbon is found in small quantities in fish liver oils and in other natural sources of vitamin A. Quite frequently it is also produced as a by-product in most of the synthetic processes of vitamin A.

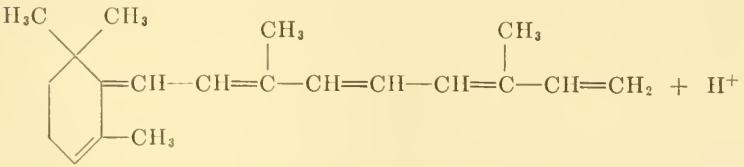
Originally[174] this hydrocarbon was thought to have a dicyclic structure, but later researches[175-177] demonstrated its monocyclic nature and the presence of six double bonds in conjugation. The most reasonable structure of this hydrocarbon is that represented by formula XCIV. A plausible mechanism of the reaction is that which assumes the formation of two carbonium ion intermediates, XCII and XCIII.[154, 178]



XCII



XCIII



XCIV Anhydrovitamin A

Anhydrovitamin A is best prepared by the method of Shantz *et al.*,[176] who crystallized it from petroleum ether at −70° to give orange prisms, m.p. 76 to 77°. A slightly different method is described by Karrer and Schwyzer.[177] The ultraviolet spectrum (Fig. 10) shows three peaks[133b, 176] at 351, 371, and 392 mμ with $E^{1\%}_{1\,cm.}$ values of 2540, 3680, and 3200, respectively. The antimony trichloride reaction shows a single band with a maximum at 620, with an $E^{1\%}_{1\,cm.}$ value of 5500, a slightly higher value than

[174] J. R. Edisbury, A. E. Gillam, I. Heilbron, and R. A. Morton, *Biochem. J.* **26**, 1164 (1932).

[175] P. Meunier, R. Dulou, and A. Vinet, *Bull. soc. chim. biol.* **25**, 371 (1942); *Compt. rend.* **216**, 907 (1943).

[176] E. M. Shantz, J. D. Cawley, and N. D. Embree, *J. Am. Chem. Soc.* **65**, 901 (1943).

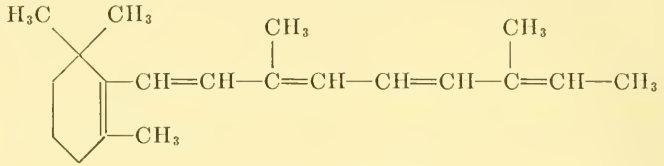
[177] P. Karrer and R. Schwyzer, *Helv. Chim. Acta* **31**, 1055 (1948).

[178] P. Meunier, *Compt. rend.* **227**, 206 (1948).

that of the all-*trans* vitamin A_1 (p. 31). The biopotency of anhydrovitamin A is about 17,000 I.U. per gram and may not be significant in view of its conversion by the rat to rehydrovitamin A.[179]

Prolonged treatment of vitamin A with ethanolic hydrogen chloride gives isoanhydrovitamin A,[133b, 175, 180] which also shows a fine structure in the ultraviolet with maxima at 300, 347, and 367 mμ, and with $E^{1\%}_{1\,cm.}$ values of 1100, 1360, and 1100, respectively. The antimony trichloride color shows a maximum at 620 mμ with an $E^{1\%}_{1\,cm.}$ value of 3200. The biological activity of isoanhydrovitamin A is of the same order of magnitude as that of anhydrovitamin A. A product which contains chlorine was also isolated.[180]

An isomer, XCV, of anhydrovitamin A, named axerophthene, was synthesized by Karrer and Benz[181] from C_{18}-ketone and ethyl magnesium



XCV Axerophthene

bromide. This also has a fine structure in the ultraviolet with maxima at 331, 346, and 364 mμ, and with $E^{1\%}_{1\,cm.}$ values of 1080, 1260, and 952, respectively. The antimony trichloride color shows two maxima at 474 and 577 mμ, respectively. The biopotency of this hydrocarbon is about 0.1 that of vitamin A.[182]

7. Vitamin A_2

a. Isolation

When fresh-water fish liver oils were examined by means of the Carr-Price reaction, two well-defined maxima were observed: one at 620 mμ attributed to vitamin A_1, and another at 693 mμ which was assigned to a new vitamin labeled vitamin A_2.[183, 184] Although the vitamin has been isolated in the pure form from selected pike liver oils,[185] it has not yet been crystallized. The ultraviolet spectrum of vitamin A_2 as compared with that of vitamin A_1 is shown in Fig. 11. Table XIV shows some of the properties of vitamin A_2 and some of its derivatives.

[179] E. M. Shantz, *J. Biol. Chem.* **182**, 515 (1950).
[180] P. Meunier, A. Guérillot-Vinet, J. Jouanneteau, and M. Gourevitch, *Compt. rend.* **226**, 128 (1948).
[181] P. Karrer and J. Benz, *Helv. Chim. Acta* **31**, 1048 (1948); **32**, 232 (1949).
[182] H. von Euler and P. Karrer, *Helv. Chim. Acta* **32**, 461 (1949).
[183] E. A. Lederer and V. A. Rozanova, *Biokhimiya* **2**, 293 (1937).
[184] A. E. Gillam, I. Heilbron, W. E. Jones, and E. Lederer, *Biochem. J.* **32**, 405 (1949).
[185] E. M. Shantz, *Science* **108**, 417 (1948).

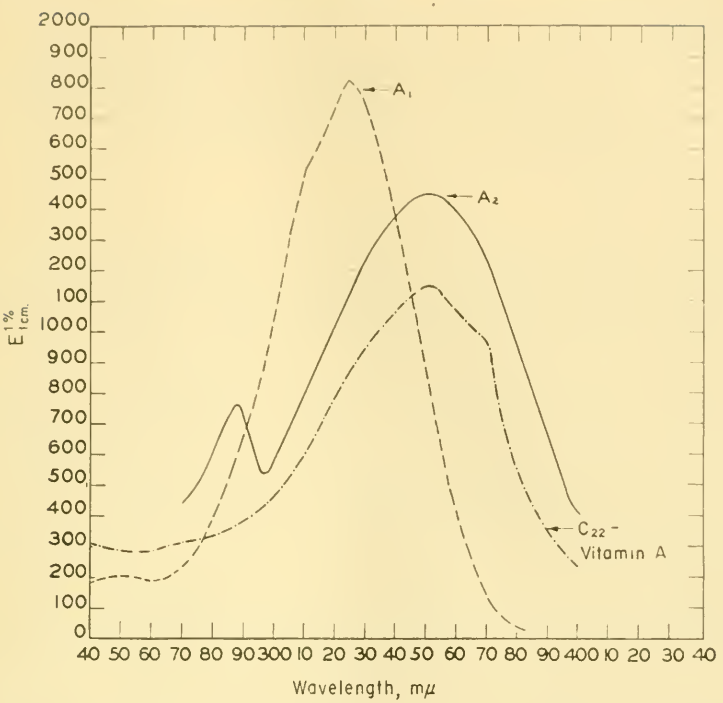
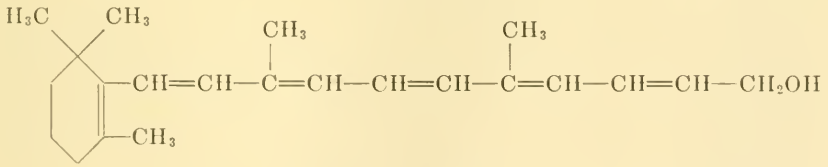


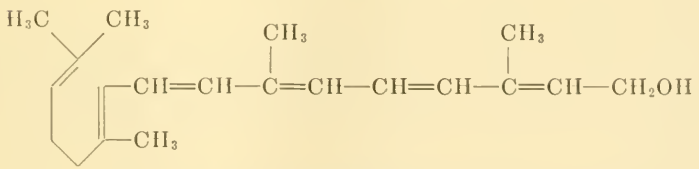
FIG. 11. Ultraviolet absorption spectra of vitamin A_1, vitamin A_2, and C_{22}-vitamin A.

b. Structure

Of all the structures proposed[184, 186, 192-196] for vitamin A_2, structures XCVI, XCVII, and XCVIII will be considered. Structure XCVI was first

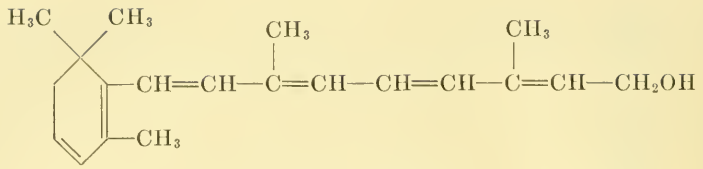


XCVI C_{22}-Vitamin A



XCVII Open-chain formula

[186] P. Karrer and P. Schneider, *Helv. Chim. Acta* **33**, 38 (1950).



XCVIII Vitamin A_2

suggested [184] to account for the shift of the absorption spectrum of vitamin A_2 toward longer wavelengths. A vitamin A of this structure has now been synthesized[188] following the routes outlined in Fig. 7, except that 3-methylheptadien-4,6-yn-1-ol-3 (XCIX) and 3-methylheptadien-3,5-yn-1-ol-7 (C) were used instead of LXIX and LXVI. Although the pure C_{22}-vitamin A has its main maximum at the same position as vitamin A_2, the second maximum at 287 mμ is absent, and the maximum of the antimony trichloride color occurs at 642 mμ instead of at 693 mμ, the maximum of vitamin A_2. From a study of the relative volatilities of natural vitamin A_1 and vitamin A_2, Gray and Cawley[192] found that the two vitamins must have nearly the same molecular weight and suggested the simple dehydrovitamin A_1 structure, XCVIII, for vitamin A_2. This was later supported by Morton *et al.*,[193] although Karrer and his students[194] supported the open-chain structure, XCVII, on the basis of their ozonolysis experiments which yielded acetone in appreciable amounts. The open-chain structure was also supported by Meunier,[196] who claims to have obtained from the oxidation of lycopene (see Table IV, p. 12) with manganese dioxide an aldehyde which had growth-promoting properties. On ozonolysis of a purer sample of vitamin A_2, Karrer and Schneider[186] subsequently failed to obtain acetone and therefore supported structure XCVIII. This structure was finally confirmed by synthesis[173] and by the preparation from the synthetic vitamin of the various derivatives shown in Table XIV.

Starting from dehydro-β-ionone (CI)[197] and building up the side chain

[187] M. K. Salah and R. A. Morton, *Biochem. J.* **43**, LVI (1948); also, private communication to E. R. H. Jones by R. A. Morton in ref. 173.

[188] N. A. Milas and D. D. Grassetti, Paper presented before the 2nd Intern. Congr. Biochem., Paris. July, 1952.

[189] E. M. Shantz and J. H. Brinkman, *J. Biol. Chem.* **183**, 467 (1950).

[190] G. Wald, *Science* **109**, 482 (1949).

[191] G. Wald, *Biochem. and Biophys. Acta* **4**, 215 (1950).

[192] E. LeB. Gray and J. D. Cawley, *J. Biol. Chem.* **131**, 317 (1939); **134**, 397 (1940).

[193] R. A. Morton, M. K. Salah, and A. L. Stubbs, *Nature* **159**, 744 (1947).

[194] P. Karrer, A. Geiger, and E. Bretscher, *Helv. Chim. Acta* **24**, 161 E (1941); **26**, 1758 (1943).

[195] L. Fieser, *J. Org. Chem.* **15**, 930 (1950).

[196] P. Meunier, *Rev. intern. vitaminol.* **23**, 21 (1951).

[197] H. B. Henbest, *J. Chem. Soc.* **1951**, 1074.

TABLE XIV

PHYSICAL PROPERTIES OF VITAMIN A_2 AND RELATED COMPOUNDS

Compound	M.p.	λ_{max}, mμ	$E\,^{1\%}_{1\,cm.}$	Solvent	References
Vitamin A_2	—	351 (352) 287 (288)	1460 (1454) 820 (771)	Ethanol	185 (173)
$SbCl_3$ color in $CHCl_3$	—	693 (693)	4100 (4000)	Chloroform	185 (173)
p-Phenylazobenzoate	76–77° (78–90°) 94–95°	341 (341) —	1190 (1200) —	Ethanol — —	185 (173) 186
Vitamin A_2 aldehyde (retinene$_2$)	77–78° (77–78°)	400 315[a]	1380 (1350) — (410)	Ethanol	187 (173)
$SbCl_3$ color in $CHCl_3$	—	730 → 705	3720 (3750)	Chloroform	187 (173)
Oxime[b]	141–143°	377.5	1790	Ethanol	(173)
2,4-Dinitrophenylhydrazone[c]	197–199°	462	1000	Ethanol	(173)
Anhydrovitamin A_2	89.5° (87–88°)	352 (352.5) 370 (370) 391 (391.5)	2040 (2000) 2980 (3040) 2620 (2740)	Ethanol	185 (173)
$SbCl_3$ color in $CHCl_3$	—	693	4400 (4500)	Chloroform	185 (173)
C_{22}-Vitamin A	—	351 370[a]	1200 550	Ethanol	188
$SbCl_3$ color in $CHCl_3$	—	642	—	Chloroform	188

[a] Inflection.

[b] Oxime of retinene$_1$, m.p. 134 to 136°.[173]

[c] 2,4-Dinitrophenylhydrazone of retinene$_1$, m.p. 214 to 216°.[173]

The biopotency of vitamin A_2 has been given as 40%[139] and 30%[173] respectively, of the biopotency of the all-*trans* vitamin A. The role of vitamin A_2 and retinene$_2$ in visual processes has been reported by Wald.[190, 191]

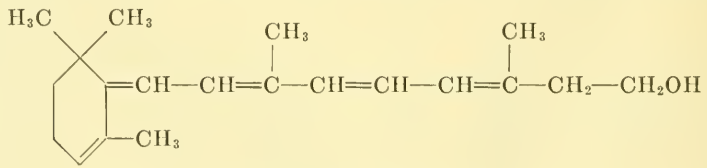
in accordance with the methods outlined in Fig. 6 failed to give satisfactory yields of the desired intermediate products, owing perhaps to the inherent limitations of these methods as discussed elsewhere. The most successful method utilized the C_{20}-vitamin A_1 acid methyl ester (LVI, m.p. 55 to 56°) as the starting material. This was allowed to react at 0° in chloroform with N-bromosuccinimide, thereby replacing one of the hydrogen atoms of carbon 3′ with bromine. Dehydrobromination of the unstable bromo compound was effected by heating with 4-phenylmorpholine. After separation and chromatography, a 25% over-all yield of the crystalline C_{20}-vitamin A_2 acid methyl ester (m.p. 45 to 47°) was obtained. This was readily converted to vitamin A_2 with lithium aluminum hydride. Owing to the instability of vitamin A_2, it was necessary to add small amounts of α-tocopherol to the final crude product before it was isolated in the pure form.

A preliminary announcement was made recently[198] of an alternative synthesis of vitamin A_2 based on the condensation of 2,6,6-trimethyl-1-ethynylcyclohexen-2-ol-1 (CII) with the ketone LXXVII via the Grignard reaction and following the sequence of reactions shown in Fig. 8.

Vitamin A_2 undergoes the same reactions as vitamin A_1, so that the derivatives shown in Table XIV have been prepared by following analogous procedures. Like vitamin A_1, vitamin A_2 is expected to exist in four stereoisomeric forms, but no work has been published along these lines. Vitamin A_2 has not yet been crystallized and may therefore be a mixture of stereoisomeric forms.

8. Rehydrovitamin A[179]

When vitamin A-deficient rats were fed with crystalline anhydrovitamin A, a growth-promoting vitamin was isolated from their livers which was neither vitamin A nor anhydrovitamin A. The ultraviolet spectrum of this vitamin had three maxima at 330, 351, and 369 mμ, respectively, and the antimony trichloride color had a maximum at 612 mμ. Its biopotency was about one-fifteenth the potency of vitamin A_1. Shantz assigned the tentative structure CIII to this vitamin. Since no degradative studies were done on this vitamin, it is highly doubtful whether it has structure CIII



CIII Rehydrovitamin A(?)

[198] N. A. Milas, P. Davis, and D. D. Grassetti, Paper presented before the 2nd Intern. Congr. Biochem., Paris. July, 1952.

and still show vitamin A activity. Retrovitamin A methyl ether (CIV),[153] which is assumed to be the methyl ether of CIII, has only a trace of biological activity. Its spectrum shows two maxima at 348 and 367 mμ, with $E^{1\%}_{1\text{ cm.}}$ values of 2190 and 1770, respectively. It also gives no isogeronic acid on ozonolysis, confirming the structure assigned to it. *allo*-Vitamin A ethyl ether (CV, m.p. 28 to 30°)[199] has essentially the same spectrum (λ_{max} 330, 348, and 367 mμ; $E^{1\%}_{1\text{ cm.}}$ values 1690, 1830, and 1520, respectively) as rehydrovitamin A, but on ozonolysis it yields geronic acid, showing the presence of a β-ionone ring.

9. Synthetic Homologs and Analogs of Vitamin A and Related Products

It would be beyond the scope of this section to discuss the synthesis of each of the various homologs and analogs of vitamin A and related products which have been found to be biologically active. Table XV lists these products together with some of their physical and biological properties.

10. Chemical Constitution and Biological Activity

From the biological potency of the various compounds given in Tables X, XII, XIII, XIV, and XV, one may derive certain generalizations. To have a high vitamin A activity a compound must have: (1) a 2′,6′,6′-trimethylcyclohexen-1′-yl or β-ionone ring; (2) a side chain of at least eleven carbon atoms containing four double bonds in conjugation with one another and with the double bond in the ring and attached to the ring in carbon atom 1′; (3) the two methyl groups in the side chain should be attached to carbon atoms 3 and 7; (4) the terminal group can be an hydroxyl, carbonyl (aldehyde), or carboxyl (in the form of its sodium salt); (5) all double bonds in the side chain must have a *trans* configuration as in structure LXXXVIII. A slight modification of this structure has the tendency to diminish or even destroy the biological activity of the compound. For example, replacing one of the *trans* double bonds with a *cis* double bond (neovitamin A) or with a triple bond (see Table XV) diminishes the biopotency of the compound. Moreover, if the methyl groups in the ring or side chain are replaced with hydrogen atoms, or attached to some other carbon atoms, the new compounds have very little or no biological activity.

It has also been found that when one of the double bonds in the side chain is hydroxylated the resulting compound is highly toxic and acts as an antivitamin A. This toxic compound was produced when β-carotene or

[199] N. A. Milas, E. Sakal, J. T. Plati, J. T. Rivers, J. K. Gladding, F. X. Grossi, Z. Weiss, M. A. Campbell, and H. F. Wright, *J. Am. Chem. Soc.* **70**, 1597 (1948).

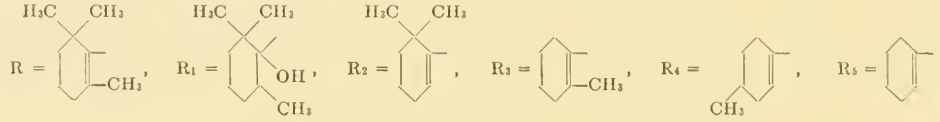
TABLE XV

HOMOLOGS AND ANALOGS OF VITAMIN A AND RELATED PRODUCTS

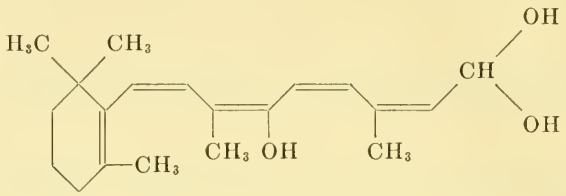
Homolog	M.p.	λ_{max}, mμ	$E^{1\%}_{1\ cm.}$	Relative potency (vitamin A = 1)	References
R—CH=CH—C(CH$_3$)=CH—CH=CH—C(CH$_3$)=CH—CH$_2$—CH$_2$OC$_2$H$_5$	—	328 367[a]	1700 900	0.01–0.02	200
R—CH=CH—C(CH$_3$)=CH—CH=CH—C(CH$_3$)=CH—CH$_2$—CH$_2$OAc	—	325 310[a]	1575 1360	0.2 –0.25	201
Analog					
R—CH=CH—C(CH$_3$)=CH—CH=CH—C(CH$_3$)=CH—CH$_2$N(CH$_3$)$_2$	—	323–324	1200	Appreciable activity	103
R—CH=CH—C(CH$_3$)=CH—C≡C—C(CH$_3$)=CH—CH$_2$OCH$_3$	—	322	1600	0.1–0.15	199
R—C≡C—C(CH$_3$)=CH—CH=CH—C(CH$_3$)=CH—CH$_2$OH	—	328 252	1230 680	0.1–0.4	154
R—C≡C—C(CH$_3$)(OH)—CH=CH—CH=C(CH$_3$)—CH=CH$_2$	—	281 271	1260 1210	0.04	154
R$_1$—C≡C—C(CH$_3$)=CH—CH=CH—C(CH$_3$)=CH—CH$_2$OH	—	317 302 226	1290 1570 270	0.01	154
R—CH=CH—C(CH$_3$)=CH—CH=CH—CH=CH—CH$_2$OAc (25% purity)	—	229 243 281 324	270 270 420 400	0	136
R—CH=CH—C(CH$_3$)=CH—CH=CH—CH=CH—C(CH$_3$)HOAc (15% purity)	—	326 223	270 350	0	136

R—CH=CH—C(CH$_3$)=CH—CH=CH—CH$_2$OH	76°	320 337	1740 1170	0.001	202
—CH=CH—C(CH$_3$)=CH—CH=CH—C(CH$_3$)=CH—CH$_2$OH	137°	330	1940		
		343 362	2100 1870	0	203
Vitamin A-Acid Analog					
R—C≡C—C(CH$_3$)=CH—CH=CH—C(CH$_3$)=CH—COOH	161°	351 265	1120 410	0.005-0.01[b]	154
R$_2$—C≡C—C(CH$_3$)=CH—CH=CH—C(CH$_3$)=CH—COOH	171°	351 258	1250 530	0.001	204
R$_3$—C≡C—C(CH$_3$)=CH—CH=CH—C(CH$_3$)=CH—COOH	167°	338	1150	0	204
R$_4$—C≡C—C(CH$_3$)=CH—CH=CH—C(CH$_3$)=CH—COOH	206°	339	1200	0	205
R$_5$—C≡C—C(CH$_3$)=CH—CH=CH—C(CH$_3$)=CH—COOH	179°	339 260	1075 470		
		257	430	0.001	204, 206
	153°	342 260	1150 480		
R$_5$—C≡C—CH=CH—CH=CH—C(CH$_3$)=CH—COOH	168°	338	1860	0.001	207

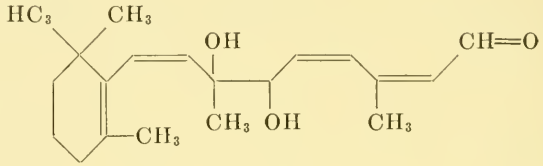
[a] Inflection.
[b] Sodium salt gives a relative activity of 0.1.



vitamin A was oxidized with vinadium tetroxide (V_2O_4).[208–210] Apparently retinene$_1$ is first formed, which is subsequently hydroxylated to give anti-vitamin A. The ultraviolet spectrum of this product shows two maxima at 290 and 340 mμ, respectively, and its antimony trichloride color shows a maximum at 545 mμ. Meunier[210] has proposed two structures (CVI and CVII) for this product.



CVI



CVII

Finally, mention should be made here of an unknown substance with vitamin A activity obtained from lard by high-vacuum distillation.[211, 212] Apparently the identity of this substance is still unknown.

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[200] N. A. Milas, S. W. Lee, C. Schuerch, Jr., R. O. Edgerton, J. T. Plati, F. X. Grossi, Z. Weiss, and M. A. Campbell, *J. Am. Chem. Soc.* **70**, 1591 (1948).

[201] N. A. Milas and M. T. Burgess, Paper presented before the 2nd Intern. Congr. Biochem., Paris. July, 1952.

[202] G. W. H. Cheeseman, I. Heilbron, E. R. H. Jones, and B. C. L. Weedon, *J. Chem. Soc.* **1949**, 3120.

[203] B. C. L. Weedon and R. J. Woods, *J. Chem. Soc.* **1951**, 2687.

[204] I. Heilbron, E. R. H. Jones, D. G. Lewis, and B. C. L. Weedon, *J. Chem. Soc.* **1949**, 2023.

[205] J. B. Toogood and B. C. L. Weedon, *J. Chem. Soc.* **1949**, 3123.

[206] I. Heilbron, E. R. H. Jones, and R. W. Richardson, *J. Chem. Soc.* **1949**, 287.

[207] I. Heilbron, E. R. H. Jones, D. G. Lewis, R. W. Richardson, and B. C. L. Weedon, *J. Chem. Soc.* **1949**, 742.

[208] P. Meunier, J. Jouanneteau, and R. Ferrando, *Compt. rend.* **230**, 140 (1950).

[209] P. Meunier, G. Zwingelstein, J. Jouanneteau, and R. Mallein, *Compt. rend.* **230**, 1323 (1950).

[210] P. Meunier, *Fortschr. Chem. org. Naturstoffe* **9**, 102 (1952).

[211] H. Kaunitz and C. A. Slanetz, *J. Nutrition* **42**, 375 (1950).

[212] H. Kaunitz and C. A. Slanetz, *Proc. Soc. Exptl. Biol. Med.* **75**, 322 (1950).

III. Biochemical Systems

GEORGE WALD

The metabolism of vitamin A involves three kinds of problem. First in importance is its general function in the tissues. Since a mammal deprived of vitamin A stops growing and eventually dies, it can be assumed that this vitamin plays some fundamental role in cellular metabolism or structure, a role perhaps particularly associated with epithelial cells, since these appear to undergo the earliest changes in vitamin A deficiency.

Next there is what may be called the vegetative metabolism of vitamin A—its formation from carotenoid precursors, storage and mobilization, absorption, and transport.

Finally, there is the specific function of vitamin A in vision. In the totality of vitamin A metabolism this would seem to be of minor importance. Two things must be said about this, however: first, that it is the only activity of vitamin A that is well understood; and second, that what has been learned of the visual processes has begun to provide a guide for pursuing the more general metabolism of vitamin A.

I shall begin therefore by reviewing the visual biochemistry of vitamin A, stressing particularly those reactions which find some scope outside the retina. Then I shall try to indicate against this background the lines of a general approach to vitamin A metabolism.

A. RHODOPSIN, PORPHYROPSIN, AND IODOPSIN[1a, b]

The retinas of most vertebrate animals contain two kinds of light receptor: rods, concerned with vision in dim light; and cones, the organs of vision in bright light and color vision. Each contains a light-sensitive pigment, rhodopsin or porphyropsin in the rods, iodopsin in the cones. All three substances are carotenoid proteins, proteins bearing carotenoid prosthetic groups to which they owe their color and sensitivity to light.

Rhodopsin is a bright-red pigment, found typically in the rods of marine fishes and land vertebrates. On exposure to light it bleaches over orange intermediates ("transient orange") to a yellow product ("visual yellow"; "indicator yellow"), and eventually to colorlessness ("visual white").

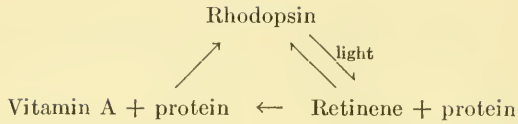
Some years ago these changes in appearance were found to correspond with a cycle of chemical changes, formulated originally as follows:[2]

[1a] The biochemistry of visual systems and their distribution among animals have been reviewed in detail by G. Wald, *Vitamins and Hormones* **1**, 195 (1943).

[1b] G. Wald, *Harvey Lectures* **41**, 117 (1945–1946).

[1c] G. Wald, *Documenta Ophthalmologica* **3**, 94 (1949).

[2] G. Wald, *J. Gen. Physiol.* **19**, 351, 781 (1935–1936).



Rhodopsin bleaches in the light over orange intermediates to a mixture of the yellow carotenoid, retinene, and a protein which we now call opsin.

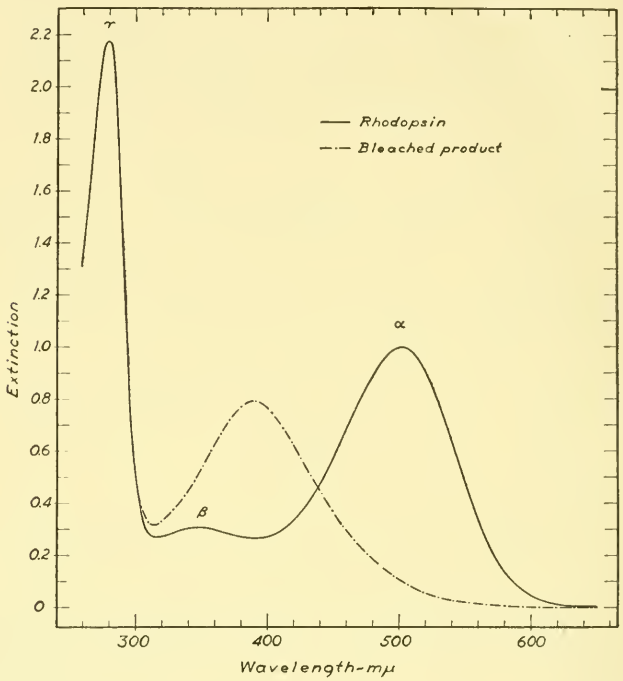


Fig. 12. Spectra of rhodopsin and of the product of its bleaching in aqueous solution. Bullfrog rhodopsin in solution in 2% aqueous digitonin at pH 5.55. Rhodopsin possesses three absorption maxima: the α band, which is mainly responsible for the spectral sensitivity of rod vision; the β band which together with the α band belongs to the carotenoid prosthetic group; and the high γ band, which is due to the protein, opsin. On bleaching in solution the α and β bands are replaced by the absorption spectrum of retinene; the protein band remains unchanged. (From G. Wald.[1c])

In the usual solutions of rhodopsin these are the final products of bleaching (Fig. 12). In the retina, however, and in the enzyme systems to be described below, retinene is converted to the colorless vitamin A. Both vitamin A and retinene recombine with opsin to regenerate the visual pigment (Fig. 13).

In the rods of fresh-water fishes, lampreys, and certain amphibians, another light-sensitive pigment is found. This, because of its purple color,

is called porphyropsin. It engages in a system of reactions exactly parallel with the rhodopsin cycle, but involving other carotenoids.[3] In the porphyropsin system, retinene is replaced by retinene$_2$, and vitamin A by vitamin A$_2$ (Fig. 13). The enzymes of both systems are identical, however, and opsin itself appears to be interchangeable in both systems. In virtually every detail of its behavior the porphyropsin system mimics rhodopsin.

The first light-sensitive pigment of cone vision was discovered in the chicken retina in 1937.[4] It has lately been shown to take part in a system of chemical reactions which differs from the rhodopsin system only in that it involves a new protein. The carotenoids of both systems are identical.[5] As in the case of the heme proteins, in which the linkage of a single pros-

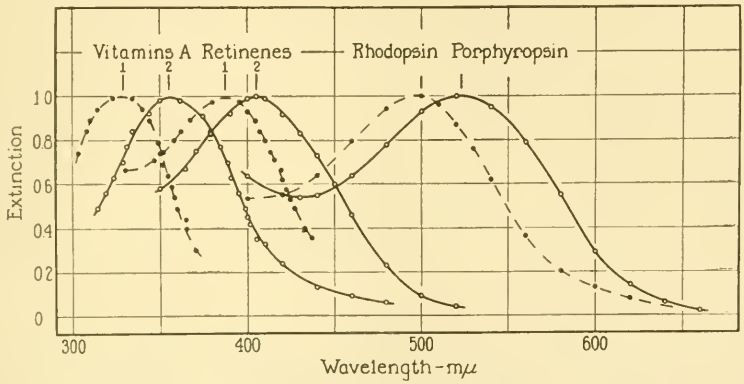


FIG. 13. Components of the rhodopsin and porphyropsin systems. Absorption spectra of crude preparations from retinas of the fresh-water calico bass (solid lines) and the marine scup (broken lines). Rhodopsin and porphyropsin are dissolved in 1% aqueous digitonin, the retinenes and vitamins A in chloroform. All maxima have been brought to the same height to facilitate comparison. (From G. Wald.[3])

thetic group, iron protoporphyrin,to different proteins determines whether the end result is hemoglobin, catalase, or peroxidase, so here it is opsin that decides whether one obtains rhodopsin or iodopsin. Actually either rod or cone opsin—as indeed hemoglobin—is not a single protein, but a family of proteins, the individuals of which vary from species to species. The family of rod proteins, since they have to do with so-called scotopic vision, may be called scotopsins; those of cone vision, photopsins.

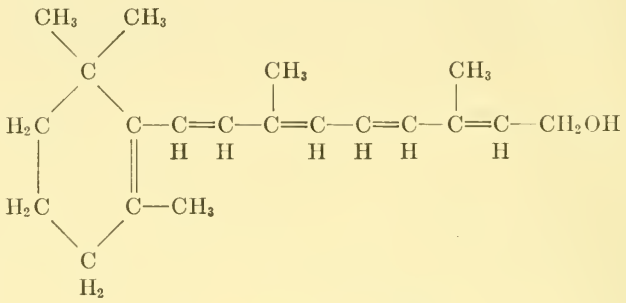
The rhodopsin and porphyropsin systems, therefore, differ only in their carotenoids, the rhodopsin and iodopsin systems only in their proteins. The visual systems are otherwise remarkably homogeneous in structure and content; what is said of one of them very nearly applies to all. The

[3] G. Wald, *J. Gen. Physiol.* **22**, 775 (1938–1939).
[4] G. Wald, *Nature* **140**, 545 (1937).
[5] G. Wald, P. K. Brown, and P. H. Smith, *Federation Proc.* **11**, 304 (1952).

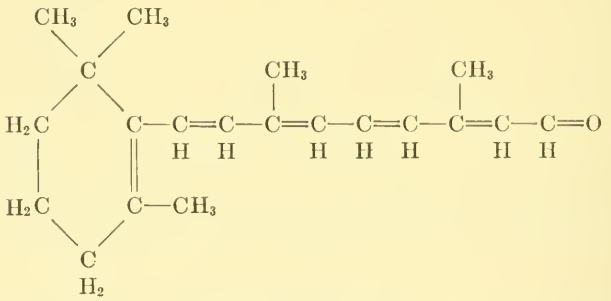
discussion which follows will concentrate primarily upon the rhodopsin system, which has led the way in all retinal chemistry. The other visual systems will be considered only as minor variations upon this central theme.

B. VITAMIN A AND RETINENE

As already noted, the bleaching of rhodopsin is a complex process. It begins with a typical photochemical reaction, which converts rhodopsin to a highly unstable, orange-red product called lumi-rhodopsin. In the dark,



Vitamin A_1, $C_{20}H_{29}OH$



Retinene$_1$, $C_{20}H_{28}O$

FIG. 14. Structural formulas of vitamin A_1 and retinene$_1$.

lumi-rhodopsin continues to react, going over with little change in color to meta-rhodopsin. Then, given access to water, meta-rhodopsin bleaches in the dark to a mixture of retinene and opsin.[6]

We owe the identification of retinene to Morton and his coworkers. Vitamin A is the primary alcohol, $C_{19}H_{27}CH_2OH$. Morton has shown that retinene is vitamin A aldehyde, $C_{19}H_{27}CHO$.[7] The structures of these substances are shown in Fig. 14; their properties are listed in Table XVI.

Morton showed also how to prepare retinene by the mild oxidation of vitamin A. His simplest procedure was to add a pinch of manganese dioxide

[6] G. Wald, J. Durell, and R. C. C. St. George, *Science* **111**, 179 (1950).

[7] S. Ball, T. W. Goodwin, and R. A. Morton, *Biochem. J.* **42**, 516 (1948).

powder to a solution of vitamin A in petroleum ether, and to leave this in the refrigerator for several days. At the end of this period the vitamin A had been converted almost entirely to retinene.[7]

TABLE XVI

PROPERTIES OF THE VITAMINS A AND RETINENES

The properties shown are those of the crystalline substances, except in the case of vitamin A_2, not yet crystallized. Each of these substances has been shown to have identical properties, whether isolated from natural sources or prepared by total synthesis. The properties given for the crystalline substances refer primarily to the prevalent all-*trans* stereoisomer. λ_{max} is the wavelength of maximum absorption; $E^{1\%}_{1\ cm.}$ is the extinction of a 1% solution, weight by volume, measured in a layer 1 cm. in depth.

Property	Vitamin A_1[8]	Retinene$_1$[7, 9]	Vitamin A_2[10–12]	Retinene$_2$[12–14]
λ_{max} in ethanol, mμ	325	383	352	400
$E^{1\%}_{1\ cm.}$ in ethanol	1800	1510	1460	1400
Antimony chloride product				
λ_{max}	620	664	693	735
$E^{1\%}_{1\ cm.}$	4400	3800	4050	4000
Melting point	64°	68°	—	77–78°
U.S.P. units per gram	3,300,000	—	1,300,000	—

On re-examining this reaction, we found it to take the following course. Vitamin A is adsorbed very strongly on manganese dioxide and is oxidized in the adsorbed state to retinene. Retinene, however, is much less strongly adsorbed and so is displaced from the manganese dioxide surface by the remaining vitamin A as fast as it is formed. In this way all the vitamin A passes over the surface of the adsorbent and is replaced by retinene in the solution.[15]

Once this mechanism was understood, the process was modified accordingly. All that is needed is to pack a small amount of manganese dioxide

[8] J. G. Baxter and C. D. Robeson, *J. Am. Chem. Soc.* **64**, 2411 (1942); J. D. Cawley, C. D. Robeson, L. Weisler, E. M. Shantz, N. D. Embree, and J. G. Baxter, *Science* **107**, 346 (1948).

[9] R. Hubbard, R. I. Gregerman, and G. Wald, *J. Gen. Physiol.* **36**, 415 (1952–1953); G. Wald and P. K. Brown, *J. Gen. Physiol.* **37**, 189 (1953–1954).

[10] E. M. Shantz, *Science* **108**, 417 (1948).

[11] E. M. Shantz and J. H. Brinkman, *J. Biol. Chem.* **183**, 467 (1950).

[12] K. R. Farrar, J. C. Hamlet, H. B. Henbest, and E. R. H. Jones, *J. Chem. Soc.* **1952**, 2657.

[13] K. R. Farrar, J. C. Hamlet, H. B. Henbest, and E. R. H. Jones, *Chemistry & Industry* **1952**, 49.

[14] H. R. Cama, P. D. Dalvi, R. A. Morton, M. K. Salah, G. R. Steinberg, and A. L. Stubbs, *Biochem. J.* **52**, 535 (1952).

[15] G. Wald, *J. Gen. Physiol.* **31**, 489 (1947–1948).

powder—about 0.6 g. to oxidize 10 mg. of vitamin A—into a piece of glass tubing, so as to form a short column of the kind used in chromatography. A solution of vitamin A in petroleum ether is poured in at the top and is drawn through under light suction. An almost pure solution of retinene runs off as the filtrate.[15, 16]

I have called this process a *chromatographic oxidation.* It is an example of what Tswett, the founder of chromatography, foresaw might become a widespread class of reactions, in which a solid acts at once as adsorbent and reagent. It seems probable that such processes display a degree of specificity and molecular orientation to be found otherwise only in enzyme systems. The force of this consideration will appear in connection with another such reaction which is considered below.

C. VITAMIN A_2 AND RETINENE$_2$

The first correct characterization of vitamin A_2, and the first—and still the only—demonstration that it is a vitamin, appeared in a biochemical description of the visual systems of fresh-water fishes.[17] This paper also first characterized retinene$_2$ and described the part that both substances play in the porphyropsin system. Both substances were identified through the absorption bands which they yield in the reaction with antimony chloride, vitamin A_2 as the 696-mμ chromogen, retinene$_2$ as the 703-mμ chromogen.

The term vitamin A_2 was not used in this paper but was introduced shortly afterward on the basis of these observations.[18] A few months earlier Lederer and Rosanova[19] had described in the Russian journal *Biokhimiya* "an abnormal reaction" in the antimony chloride test with liver oils of certain fresh-water fishes. The "abnormality" consisted in the appearance of predominant absorption bands at 645 and 690 mμ. The authors assigned the main importance to the 645-mμ chromogen: "further experiments will show, whether really the chromogen 645 of fish liver oils can act as a vitamin A." Some years earlier, Heilbron *et al.*[20] had noted in antimony chloride

[16] Mr. P. K. Brown in our laboratory has found that the addition of about 2% absolute ethyl alcohol to the vitamin A solution in petroleum ether results in a purer product and an increased yield. Preparations of manganese dioxide, however, vary considerably in activity. As with all adsorbents, the physical state of the solid particles has much to do with their action. The directions given here, and given below for the oxidation of vitamin A_2, hold for the most active preparations that we have examined. With weaker preparations, larger amounts of manganese dioxide are needed.

[17] G. Wald, *Nature* **139**, 1017 (1937).

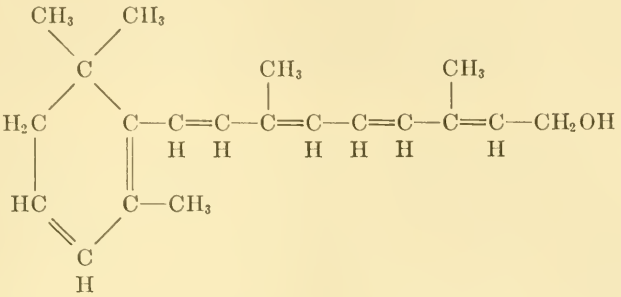
[18] E. Lederer, V. A. Rozanova, A. E. Gillam, and I. M. Heilbron, *Nature* **140**, 233 (1937); J. R. Edisbury, R. A. Morton, and G. W. Simpkins, *Nature* **140**, 234 (1937).

[19] E. A. Lederer and V. A. Rozanova, *Biokhimiya* **2**, 2 (1937).

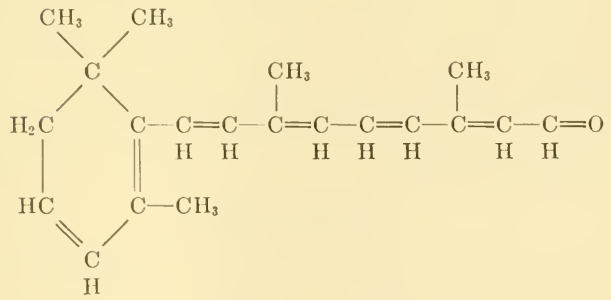
[20] I. M. Heilbron, A. E. Gillam, and R. A. Morton, *Biochem. J.* **25**, 1352 (1931).

tests with marine fish liver oils and concentrates the "appearance in certain cases of bands at 635, 645, 656, 680 and 690 mμ." It is sometimes said that the presence of the number 690 in this list constitutes the discovery of vitamin A_2; by the same token the remaining numbers should mark the discovery of vitamins A_3 through A_6.

The history of vitamin A_2 in the years following its discovery was remarkably confused. Very early it was reported to have growth-promoting activity in rats;[21] this was later denied,[22] then reaffirmed.[23, 24] It is now



Vitamin A_2, $C_{20}H_{27}OH$



Retinene$_2$, $C_{20}H_{26}O$

FIG. 15. Structural formulas of vitamin A_2 and retinene$_2$.

clear that it has such activity, although no rigorous proof has yet been presented that in the rat it acts directly, rather than as a provitamin A_1. Its ozonolysis was claimed to yield geronic acid,[21] then acetone,[22] then formaldehyde,[24] in each case indicating chemical structures, all of which have since been abandoned.

[21] A. E. Gillam, I. M. Heilbron, W. E. Jones, and E. Lederer, *Biochem. J.* **32**, 405 (1938).

[22] P. Karrer, A. Geiger, and E. Bretscher, *Helv. Chim. Acta* **24**, 161E (1941).

[23] J. L. Jensen, E. M. Shantz, N. D. Embree, J. D. Cawley, and P. L. Harris, *J. Biol. Chem.* **149**, 473 (1943).

[24] P. Karrer and E. Bretscher, *Helv. Chim. Acta* **26**, 1758 (1943).

The chemistry and biochemistry of vitamin A_2 have only lately been clarified, with its purification and the preparation of crystalline derivatives by Shantz,[10] the accurate estimation of its biological activity by Shantz and Brinkman,[11] and the total synthesis of vitamin A_2 and retinene$_2$ by Farrar *et al.*[12, 13] The latter research has established the structures shown in Fig. 15. The presence of a second double bond in the ring had been suggested earlier by Gray and Cawley[25] and by Morton *et al.*[26] Other properties of vitamin A_2 and retinene$_2$ are listed in Table XVI.

Retinene$_2$, like retinene$_1$, is easily prepared from the corresponding vitamin A by chromatographic oxidation on manganese dioxide.[26, 27] We have found it best in this process to use only about 0.3 g. of manganese dioxide to oxidize 10 mg. of vitamin A_2, half as much therefore as is used in oxidizing vitamin A_1.[16]

D. THE ALCOHOL DEHYDROGENASE SYSTEM

Whereas the usual solutions of rhodopsin yield retinene and opsin as the end products of bleaching, in the retina retinene is reduced quantitatively to vitamin A.

This process is catalyzed by a soluble enzyme system, to which we first gave the name *retinene reductase*. It consists of an enzyme protein or apoenzyme, working together with the coenzyme cozymase, coenzyme I or DPN. The latter is one of the major coenzymes of biological oxidation and reduction, the substance which in yeast reduces acetaldehyde to ethyl alcohol and in muscle reduces pyruvic acid to lactic acid. In the retina it transfers two hydrogen atoms to the aldehyde group of retinene, reducing it to the alcohol group of vitamin A:[28]

$$C_{19}H_{27}CHO + \text{DPN-H}_2 \underset{\longleftarrow}{\overset{\text{retinene reductase or alcohol dehydrogenase}}{\longrightarrow}} C_{19}H_{27}CH_2OH + \text{DPN}$$

Retinene + dihydrocozymase — Vitamin A + cozymase

In the porphyropsin system the same enzyme reduces retinene$_2$ to vitamin A_2. The enzyme proteins of both systems are interchangeable, and the coenzyme is identical in both.[29]

This enzyme system is readily assembled in solution. The protein is readily extracted with buffer solutions from homogenized retinas or from suspensions of the outer segments of the rods. Reduced cozymase is prepared by Ohlmeyer's procedure.[30] As substrate, retinene$_1$ or retinene$_2$ is

[25] E. LeB Gray and J. D. Cawley, *J. Biol. Chem.* **134**, 397 (1940).
[26] R. A. Morton, M. K. Salah, and A. L. Stubbs, *Nature* **159**, 744 (1947).
[27] G. Wald, *Biochim. et Biophys. Acta* **4**, 215 (1950).
[28] G. Wald and R. Hubbard, *J. Gen. Physiol.* **32**, 367 (1948–1949).
[29] G. Wald, *Science* **109**, 482 (1949); *Biochim. et Biophys. Acta* **4**, 215 (1950).
[30] P. Ohlmeyer, *Biochem. Z.* **297**, 66 (1938).

prepared by oxidizing the corresponding vitamin A on manganese dioxide. When these three components are mixed, the retinene is reduced almost completely to vitamin A (Fig. 16).

An enzyme called alcohol dehydrogenase has long been known to occur in a variety of animal tissues—liver, kidney, and intestine. This, with cozymase as coenzyme, catalyzes the equilibria between various alcohols and their aldehydes.[31] Shortly after the retinene reductase system was found in the retina, Bliss[32] reported that crude preparations of liver alcohol dehydrogenase catalyze the equilibrium between retinene and vitamin A. We have confirmed this observation, using crystalline alcohol dehydrogenase from horse liver.[33] Conversely it was shown that the enzyme present in frog retinas oxidizes ethyl alcohol to acetaldehyde (W. Yudkin, unpublished observations).

There is no present reason, therefore, to distinguish the retinal enzyme from the alcohol dehydrogenase found in other animal tissues. We shall refer to it hereafter by this more general designation.

Cozymase introduces a second vitamin into the chemistry of visual systems. Its active constituent is nicotinamide, the antipellagra factor of the vitamin B complex. In the retina it performs the peculiar function of helping to regenerate the vitamins A.

In the system as assembled *in vitro* there is further room for the action of vitamins. Retinal homogenates and extracts contain an enzyme which destroys cozymase, a so-called nucleosidase, which is widely distributed in animal tissues. Cozymase is protected from this attack by the presence of free nicotinamide;[34] or alternatively by α-tocopheryl phosphate.[35] When the latter is used in the present system, three vitamins cooperate in a single reaction in solution—vitamin E phosphate protects the nicotinamide complex, DPN, as it reduces retinene to vitamin A.

Alcohol dehydrogenase is the only enzyme system known to act directly upon vitamin A. It opens a broad series of relationships between the visual processes and other aspects of metabolism. In alcohol dehydrogenase a wide variety of tissues share an enzyme which permits them to negotiate reversible transformations between vitamin A and retinene; and through cozymase these processes are connected with the main pathways of cellular respiration and fermentation, in which this coenzyme plays a central role. Quite apart from the visual processes, the alcohol dehydrogenase system

[31] C. Lutwak-Mann, *Biochem. J.* **32**, 1364 (1938).
[32] A. F. Bliss, *Biol. Bull.* **97**, 221 (1949).
[33] R. Hubbard and G. Wald, *Proc. Natl. Acad. Sci. U. S.* **37**, 69 (1951).
[34] P. J. G. Mann and J. H. Quastel, *Biochem. J.* **35**, 502 (1941); P. Handler and J. R. Klein, *J. Biol. Chem.* **143**, 49 (1942).
[35] M. E. Spaulding and W. D. Graham, *J. Biol. Chem.* **170**, 711 (1947).

will probably be found to have a large place in the general metabolism of vitamin A. Its properties will therefore be reviewed here in some detail.

Two alcohol dehydrogenases are known, which with cozymase as coenzyme

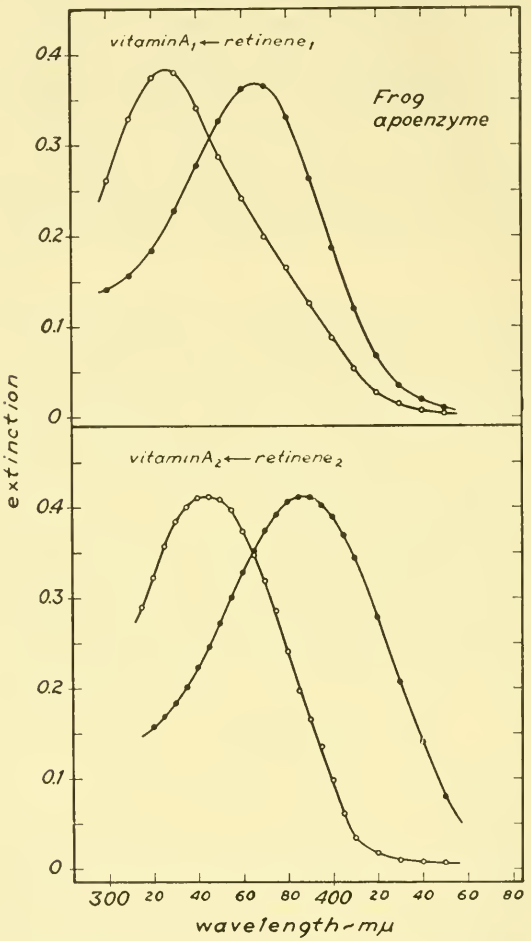


FIG. 16. The action of alcohol dehydrogenase from the frog retina on retinene$_1$ and retinene$_2$. Each preparation included retinene dissolved in 1% digitonin solution, 0.7 mg. of reduced cozymase per milliliter, 5.5 mg. of nicotinamide per milliliter, and an extract of homogenized frog retinas in *M*/30 phosphate buffer, pH 6.81. The controls differed only in that the retinal extracts were replaced either with the same extract which had been boiled for ½ minute (upper figure) or with the phosphate buffer alone (lower figure). The experimental and control mixtures were incubated for 2 hours at 23°. Methanol was added to each to a concentration of 60%, and they were extracted with hexane. The spectra of the hexane extracts are shown. Those from the controls (solid circles) show the unaltered retinenes; those from the enzyme mixtures (open circles) show complete reduction to the corresponding vitamins A. (From G. Wald.[27])

catalyze the equilibrium between ethanol and acetaldehyde. That found in animal tissues, already considered in part, was crystallized by Bonnichsen from horse liver.[36] The other, found in yeast, has been crystallized by Negelein and Wulff[37] and by Racker.[38] Unlike the animal enzyme, yeast alcohol dehydrogenase is not a catalyst for the vitamin A-retinene equilibrium.[39]

Yeast alcohol dehydrogenase is inhibited by monoiodoacetate, a poison which does not affect the animal enzyme. For this reason it is usually said that the yeast enzyme depends for its action upon sulfhydryl groups, whereas the animal enzyme does not require —SH groups. We have recently found, however, that crystalline horse liver alcohol dehydrogenase is inhibited completely by the powerful and specific sulfhydryl poison, *p*-chloromercuribenzoate (2×10^{-4} *M*). This inhibition is reversed by adding glutathione. One can be reasonably sure, therefore, that animal alcohol dehydrogenase, like that of yeast, is a sulfhydryl enzyme.[40]

The equilibrium catalyzed by the alcohol dehydrogenase system can be written:

$$\text{Alcohol} + \text{DPN} = \text{Aldehyde} + \text{DPN-H}_2$$

For this, one can write the mass action expression:

$$K = \frac{(\text{Aldehyde})(\text{DPN-H}_2)}{(\text{Alcohol})(\text{DPN})}$$

Racker[38] has measured the equilibrium between ethanol and acetaldehyde, catalyzed by yeast alcohol dehydrogenase (of course the nature of the enzyme is of no importance here, since it has no effect upon the position of the equilibrium). He found that the equilibrium constant K is 1.3×10^{-4} at pH 7. That is, in an equilibrium in which cozymase is half reduced, half oxidized, the ratio of alcohol to acetaldehyde is about 8000:1.

Racker found, however, that this equilibrium depends markedly upon pH. The logarithm of the equilibrium constant, log K, varies linearly with pH between about pH 7 and 9.6. The same is true of the lactic acid-pyruvic acid equilibrium. The reason for this relationship is clear if the equilibrium is rewritten as follows:

[36] R. K. Bonnichsen, *Acta Chem. Scand.* **4**, 715 (1950).
[37] E. Negelein and H.-J. Wulff, *Biochem. Z.* **293**, 351 (1937).
[38] E. Racker, *J. Biol. Chem.* **184**, 313 (1950).
[39] R. K. Bonnichsen, Personal communication cited in R. Hubbard and G. Wald, *Proc. Natl. Acad. Sci. U. S.* **37**, 69 (1951); A. F. Bliss, *Arch. Biochem. and Biophys.* **31**, 197 (1951).
[40] R. Hubbard and G. Wald, Unpublished observations; cited as a personal communication in H. Theorell and R. Bonnichsen, *Acta Chem. Scand.* **5**, 329 (1951).

$$\text{Alcohol} + \text{DPN}^+ = \text{Aldehyde} + \text{DPN-H} + \text{H}^+$$

$$K_{\text{H}} = \frac{(\text{Aldehyde})(\text{DPN-H})(\text{H}^+)}{(\text{Alcohol})(\text{DPN}^+)}$$

The new equilibrium constant, K_{H}, is independent of pH, and for the ethanol-acetaldehyde equilibrium it has the value 1.15×10^{-11}.[38]

The outcome of this discussion is that, the higher the alkalinity, the more the alcohol-aldehyde equilibrium is displaced in the oxidative direction, toward aldehyde. For an equilibrium condition in which DPN is half reduced, the ratio of ethyl alcohol to acetaldehyde at pH 7 is about 8000:1, at pH 8 it is about 800:1, at pH 9 about 80:1. This may be an important consideration in certain physiological conditions.

Bliss[41] has examined the equilibrium between vitamin A and retinene, catalyzed by horse liver alcohol dehydrogenase (Fig. 17). The equilibrium constant, K, depends upon pH just as described above; and again a K_{H} can be computed which is independent of pH. Bliss found K_{H} in this system to have the value 3.3×10^{-9}. That is, this equilibrium lies much further over toward oxidation—toward retinene—than does the alcohol-acetaldehyde equilibrium. In this case, when DPN is half reduced, the ratio of vitamin A to retinene at pH 6 is about 300:1, at pH 7 about 30:1, and at pH 8 about 3:1.

Another consideration may incline this equilibrium toward retinene. Theorell and Bonnichsen[42] have observed that alcohol dehydrogenase forms a complex with cozymase which has a considerably higher oxidation-reduction potential than the free coenzyme. In the presence of high concentrations of the enzyme protein, favoring formation of the complex, a stronger oxidation of alcohol to aldehyde is anticipated than is described by the above equations. Theorell and Bonnichsen believe that in liver the molar concentration of the enzyme protein approaches that of DPN, ensuring nearly complete binding of the coenzyme at neutrality. It is possible that a similar condition exists in the retina.

All these factors together make it appear that, although the vitamin A-retinene equilibrium catalyzed by animal alcohol dehydrogenase favors reduction, the disproportion between retinene and vitamin A is not extreme, and it would require little in the way of special conditions to displace the equilibrium in the other direction, toward oxidation. This consideration is of great importance in visual systems; and it may prove to play an important part also in other phases of vitamin A metabolism.

In view of what has been said it may not be clear why in the isolated retina retinene is reduced so completely to vitamin A that finally no retinene

[41] A. F. Bliss, *Arch. Biochem. and Biophys.* **31**, 197 (1951).
[42] H. Theorell and R. Bonnichsen, *Acta Chem. Scand.* **5**, 329 (1951).

at all can be distinguished. In part this behavior may be explained by the fact that the surviving tissue tends to become acid as metabolic products accumulate, so inclining the equilibrium toward reduction. The system may be thrown out of balance further by another process which has not yet been mentioned. This is the esterification of vitamin A, which proceeds

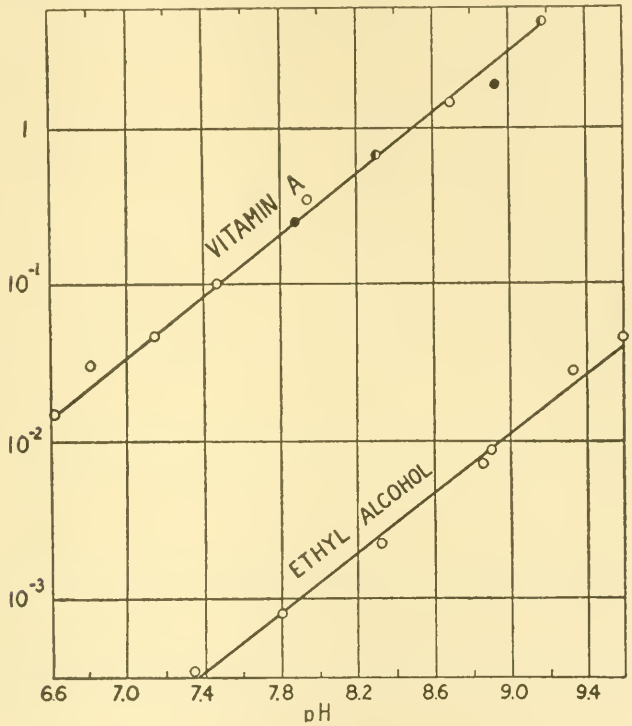


FIG. 17. The vitamin A-retinene and the ethanol-acetaldehyde equilibria as a function of pH. Ordinates show values of the equilibrium constant, K. The data on the vitamin A-retinene equilibrium are from Bliss[41] and involve the action of horse liver alcohol dehydrogenase. The data on ethyl alcohol are from Racker[38] and involve the action of crystalline yeast alcohol dehydrogenase. (From A. F. Bliss.[41])

very actively in the retina and, by removing vitamin A, may displace the equilibrium continuously in the reductive direction.

E. THE SYNTHESIS OF RHODOPSIN

We have to this point described the degradation of rhodopsin to a mixture of vitamin A and opsin. In the eye these products recombine to form the visual pigment.

Many years ago Kühne recognized that rhodopsin is synthesized in the retina in two ways: a relatively rapid "anagenesis" from yellow precursors,

which occurs to some degree in the isolated retina and even in solution; and a slower "neogenesis" from colorless precursors, which Kühne believed to occur only in the living eye and to require the cooperation of the pigment epithelium.[43] These processes can now be identified with the synthesis of rhodopsin from the yellow retinene, and from the colorless vitamin A.

Hecht *et al.*[44] and Chase and Smith[45] confirmed Kühne's observation that

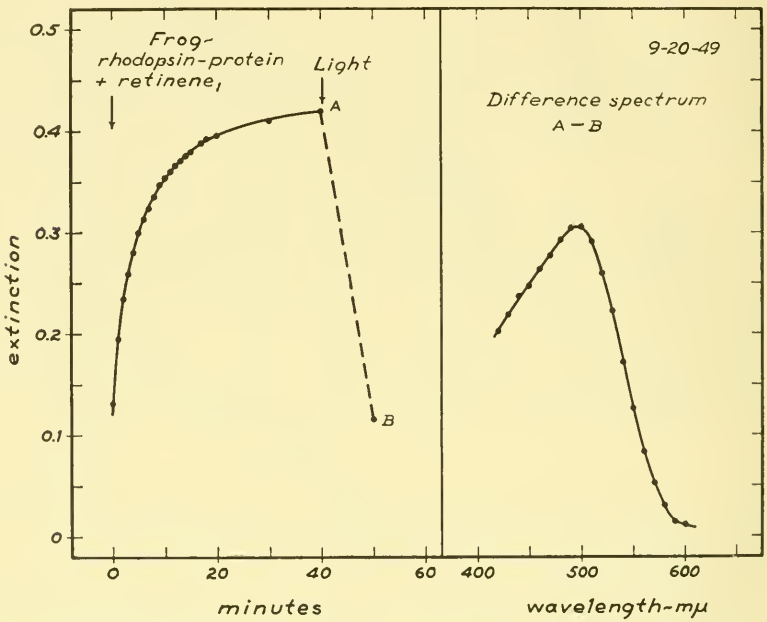


FIG. 18. The synthesis of rhodopsin from retinene and opsin. A solution of frog opsin was mixed with retinene (10 γ per milliliter), and this mixture was incubated in the dark at room temperature. The measurements at the left follow the rise in extinction at 500 mμ as rhodopsin is synthesized. At *A* the product was exposed to daylight for 20 minutes; it bleached to *B*. The difference in the absorption spectrum before and after this bleaching (*A*-*B*) is shown at the right. It has the maximum at about 498 mμ characteristic of regenerated rhodopsin (From G. Wald and P. K. Brown.[46])

rhodopsin regenerates slightly in solutions which are bleached—to retinene and opsin—and replaced in the dark. Their usual regenerations were in the neighborhood of 5 to 10%; the largest reported was 15%.

Recently we found that, when rhodopsin solutions are supplemented with

[43] W. Kühne, *in* L. Hermann: Handbuch der Physiologie, Vol. 3, Part 1, p. 321. F. C. W. Vogel, Leipzig, 1879.

[44] S. Hecht, A. M. Chase, S. Shlaer, and C. Haig, *Science* **84**, 331 (1936).

[45] A. M. Chase and Emil L. Smith, *J. Gen. Physiol.* **23**, 21 (1939–1940).

[46] G. Wald and P. K. Brown, *Proc. Natl. Acad. Sci. U. S.* **36**, 84 (1950).

high concentrations of retinene, they regenerate up to 85 % after bleaching.[46] What is more, one can extract from light-adapted rods a colorless, carotenoid-free solution of opsin, which on incubation with retinene in the dark forms a high concentration of rhodopsin (Fig. 18).

No other molecules than retinene and opsin are required for this reaction, nor does it require an external source of energy. This is a spontaneous—i.e., an energy-yielding—reaction. It is the *bleaching* of rhodopsin, its cleavage into retinene and opsin, that requires energy, usually in the form of light. Given the opportunity—i.e., removal of light—retinene and opsin reunite spontaneously to regenerate the visual pigment.

There remains the more difficult problem, the synthesis of rhodopsin from vitamin A and opsin. No such synthesis had been observed *in vitro*, and all attempts to stimulate it outside the living tissues had failed. Yet the ease with which retinene and opsin unite to form rhodopsin suggested that, if it were only possible to oxidize vitamin A to retinene, the job would be done. This proved to be true. All the difficulty in making rhodopsin from vitamin A is implicit in the difficulty of oxidizing vitamin A to retinene.

We have already noted that the equilibrium between vitamin A and retinene favors reduction rather than oxidation. In the much more unbalanced equilibrium between ethanol and acetaldehyde, however, it has long been known that the reaction can be driven in the oxidative direction by introducing an aldehyde-trapping reagent—some substance, such as cyanide, bisulfite, or semicarbazide, which by condensing with the aldehyde removes it from the system, so forcing its continuous production.

As a trapping reagent for retinene in the alcohol dehydrogenase system, we have introduced hydroxylamine, NH_2OH. This condenses rapidly with retinene at room temperature to form retinene oxime:

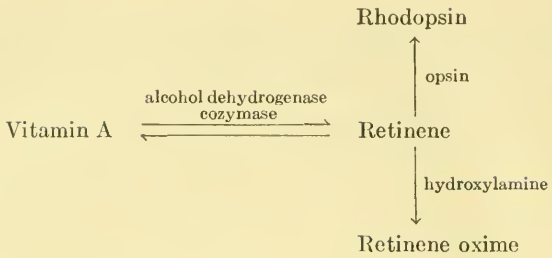
$$\underset{\text{Retinene}}{C_{19}H_{27}CHO} + \underset{\text{hydroxylamine}}{NH_2OH} \rightleftharpoons \underset{\text{Retinene oxime}}{C_{19}H_{27}CH{=}NOH} + H_2O$$

In the presence of this reagent, the alcohol dehydrogenase system oxidizes vitamin A to retinene in considerable yield.[47]

The retina, however, already contains the specific retinene-trapping reagent, opsin. Opsin should be able to substitute in the alcohol dehydrogenase system for hydroxylamine and should drive a continuous oxidation of vitamin A to retinene, by continuously removing retinene to form rhodopsin.

The trouble with this notion is that an isolated retina, bleached to colorlessness, and incubated in the dark, seems to contain all the components

[47] G. Wald and R. Hubbard, *Proc. Natl. Acad. Sci. U. S.* **36**, 92 (1950).



needed to make rhodopsin in this way, yet it does not visibly produce rhodopsin.

Careful extraction of whole frog retinas which had been bleached to colorlessness and then incubated in the dark nevertheless showed that they do form a little rhodopsin, perhaps 10 % as much as would be formed during dark adaptation *in vivo*. Retinal homogenates behave similarly. When retinal homogenates were supplemented with cozymase, the yield of rhodopsin was doubled. Kühne had believed that the synthesis of rhodopsin from colorless precursors—i.e., from vitamin A—demands the cooperation of the pigment epithelium; and it was indeed found that the addition to retinal homogenates of a homogenate of pigment epithelium doubles the yield of rhodopsin again, bringing it to about 40 %.[47]

What the pigment epithelium contributes to this synthesis is not yet wholly analyzed. One factor which it is known to contribute, however, is vitamin A. A frog retinal homogenate can be shown to make rhodopsin from vitamin A supplied by a pigment epithelium homogenate. Alternatively, the addition of free vitamin A to a retinal homogenate increases considerably the yield of rhodopsin. Another factor which helps is the addition of respiratory enzymes—for example, the particles which form the "succinoxidase" system of pig heart—which aid presumably by keeping cozymase oxidized. When all these types of supplementation were combined, yields of rhodopsin were obtained as high as 60 %.[48]

It can be concluded that all factors which promote the oxidation of vitamin A to retinene aid the synthesis of rhodopsin. The rhodopsin system can therefore be formulated as in Fig. 19. What was originally thought to be a special pathway for the synthesis of rhodopsin from vitamin A now appears to consist in the special conditions required to drive the oxidation of vitamin A to retinene.

If this view of the rhodopsin system is correct, it should be possible to assemble the entire system in solution by mixing four substances: vitamin A, alcohol dehydrogenase, cozymase, and opsin. This is correct. We have made such mixtures, using vitamin A from fish liver oils, cozymase from

[48] R. Hubbard and G. Wald, *Proc. Natl. Acad. Sci. U. S.* **37**, 69 (1951).

yeast, and alcohol dehydrogenase from horse livers. Only opsin needs to be obtained from the retina. When such a mixture is placed in the dark, it synthesizes rhodopsin (Fig. 20). Brought into the light, it bleaches. Replaced in the dark, it makes more rhodopsin. This mixture of four substances carries out in solution all the reactions of the rhodopsin system.[48]

F. *cis-trans* ISOMERS OF VITAMIN A AND RETINENE

The four-component system just described was assembled originally using a fish liver oil concentrate as the source of vitamin A. When this

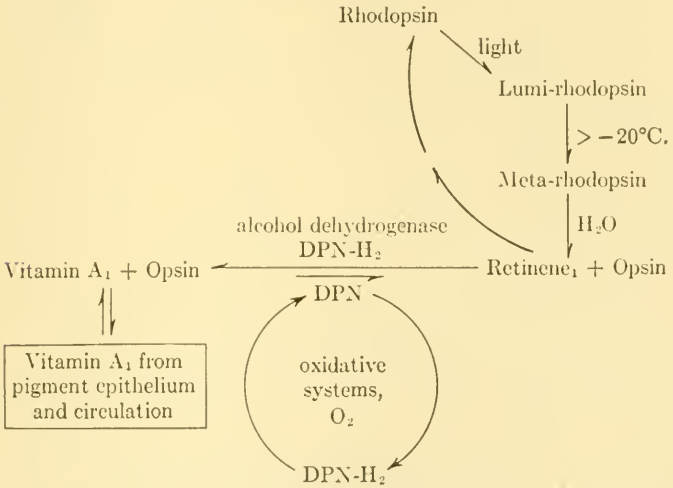


FIG. 19. Diagram of the rhodopsin system. The bulk of the system lies within the outer segments of the retinal rods, but it is supplemented with vitamin A, respiratory factors, and oxygen itself from the pigment epithelium and the blood circulation. (From G. Wald.[48a])

experiment was repeated using crystalline vitamin A, almost no rhodopsin was formed.

Such difference in behavior in two preparations of vitamin A could have only one explanation. Vitamin A, like other carotenoids, is known to exist in several different forms, geometrical or *cis-trans* isomers of one another. Most of what is known of *cis-trans* isomerization among carotenoids is due to the work of Zechmeister and his colleagues; in close association with Zechmeister's experiments, Pauling has developed the theory of the subject.[49]

According to Pauling, in such structures as the carotenoids, *cis-trans*

[48a] G. Wald, *Science* **113**, 287 (1951).

[49] L. Zechmeister, *Chem. Revs.* **34**, 267 (1944).

isomerization is possible only about the double bonds adjacent to methyl side groups. Anywhere else in the molecule the *cis* configuration is hindered sterically. Hence vitamin A and retinene, which possess two such double bonds, should exist in four stereoisomeric modifications: all-*trans*, 3-*cis*, 5-*cis*, and 3,5-di-*cis*. These are shown in Fig. 21.

Ordinary crystalline vitamin A, as well as the bulk of commercial synthetic vitamin A, is the all-*trans* isomer. Fish liver oils, however, are known to contain mixtures of all the stereoisomers of vitamin A. The observation

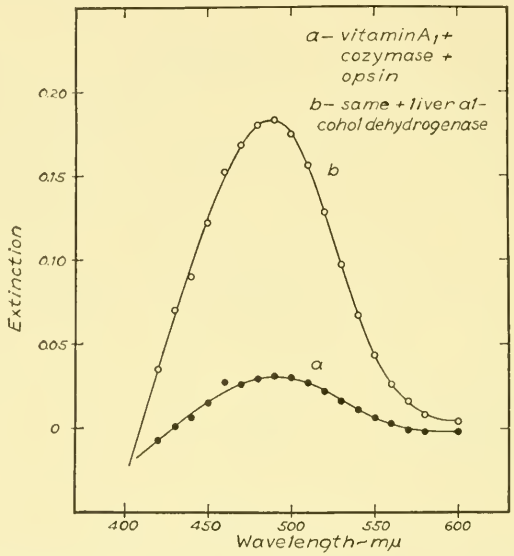


Fig. 20. Synthesis of rhodopsin by a solution of known components. The upper curve shows the difference spectrum—the difference in absorption spectrum before and after bleaching—of rhodopsin synthesized by incubating together vitamin A from fish liver oils, crystalline horse liver alcohol dehydrogenase, yeast cozymase, and frog opsin. The lower curve shows the rhodopsin formed in an identical mixture lacking only the alcohol dehydrogenase. (From R. Hubbard and G. Wald.[48])

described above means, therefore, that rhodopsin cannot be synthesized *in vitro* from all-*trans* vitamin A, but requires instead a *cis* isomer present in liver oils.

One of the *cis* isomers of vitamin A, called neovitamin A, was isolated by Robeson and Baxter.[51] For reasons which will appear below we shall refer to this hereafter as neovitamin A*a*. This substance also, on incubation in our four-component system, yields no rhodopsin.

It is well known from Zechmeister's work that any single geometrical

[50] R. Hubbard and G. Wald, *Science* **114**, 60 (1952).

[51] C. D. Robeson and J. G. Baxter, *J. Am. Chem. Soc.* **69**, 136 (1947).

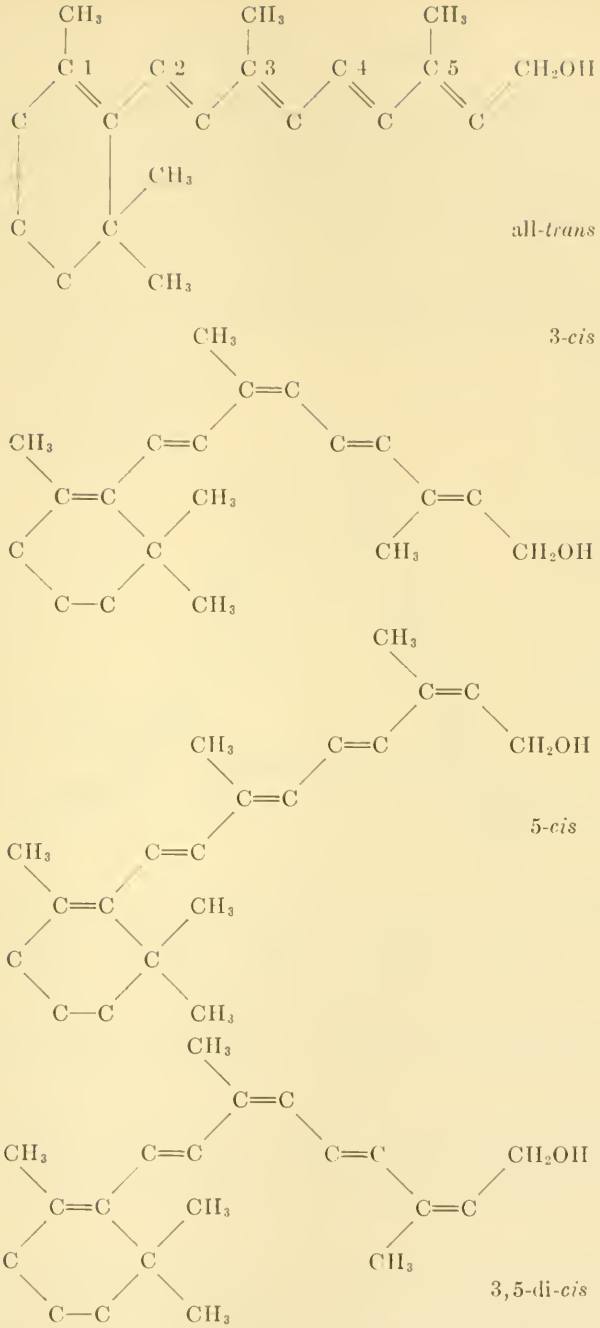


FIG. 21. Structures of four geometrical isomers of vitamin A. According to present theory, *cis-trans* isomerization occurs readily only about double bonds 3 and 5, yielding only the four isomers shown. Ordinary crystalline vitamin A, as also the bulk of the commercial synthetic product is the all-*trans* isomer. It is not yet possible to assign the other structures with confidence to specific preparations of the vitamin. (From R. Hubbard and G. Wald.[50])

isomer of a carotenoid can be isomerized to a mixture of all possible isomers by exposure to light in the presence of a trace of iodine. When this is done to crystalline all-*trans* vitamin A and neovitamin A*a*, they become as effective precursors of rhodopsin as fish liver oil concentrate (Fig. 22).

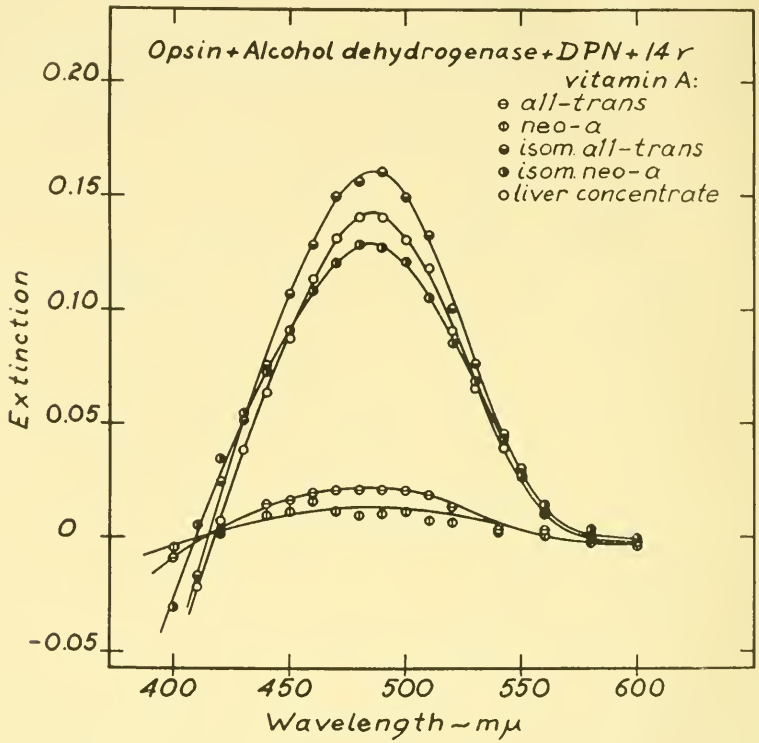


FIG. 22. The synthesis of rhodopsin from vitamin A of five different origins: crystalline all-*trans*, crystalline neovitamin A*a*, isomerized all-*trans*, isomerized neovitamin-*a* and fish liver oil concentrate. The figure shows the difference spectra of rhodopsin obtained by incubating equal amounts of each of these preparations with opsin, alcohol dehydrogenase, and cozymase. Only traces of rhodopsin were synthesized from all-*trans* or neovitamin A*a*; but after these substances had been isomerized with light in the presence of iodine, they were as effective as liver oil vitamin A in forming rhodopsin. (From R. Hubbard and G. Wald.[51a])

It is apparent, therefore, that we are dealing here with a remarkable instance of stereochemical specificity. What it involves primarily is the shape of the vitamin A molecule. All-*trans* vitamin A, as shown in Fig. 21, has a relatively straight side chain. All the other isomers are bent by their *cis* linkages in various ways. Such differences in shape may be unimportant in

[51a] R. Hubbard and G. Wald, *J. Gen. Physiol.* **36**, 269 (1952–1953).

some connections, but they are decisive for the reaction of a molecule with an enzyme or with any other type of protein.

The synthesis of rhodopsin involves two proteins and indeed is a two-step reaction. First vitamin A is oxidized to retinene by cozymase on the enzyme protein, alcohol dehydrogenase; then retinene couples with the protein opsin to form rhodopsin. The first of these reactions has proved to be relatively unspecific toward the isomers of vitamin A and retinene. It is the second reaction, the combination of retinene with opsin, that demands a specific *cis* isomer of retinene.

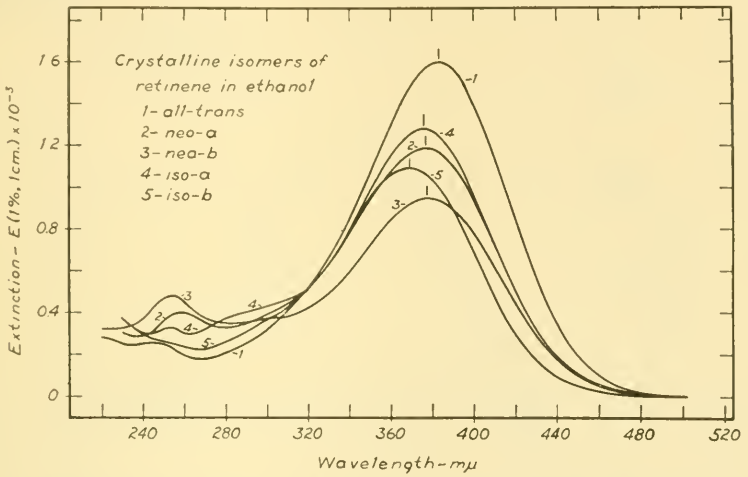


FIG. 23. Absorption spectra of crystalline stereoisomers of retinene in ethyl alcohol. The absorption maximum in each spectrum is marked with a vertical bar. Neoretinenes *a* and *b* and isoretinene *a* appear to be mono-*cis* structures; and isoretinene *b* is a di-*cis* retinene. Each presumptive *cis* linkage shifts the absorption maximum 5.5 to 7 mμ toward shorter wavelengths. All-*trans* retinene was first crystallized by Ball *et al.*;[7] neoretinenes *a* and *b* in our laboratory; and isoretinenes *a* and *b* in the Organic Research Laboratory of Distillation Products Industries. (From R. Hubbard *et al.*[9])

We have now examined five crystalline isomers of retinene: all-*trans* retinene, first prepared by Ball *et al.*[7] (1948); neoretinenes *a* and *b*, first isolated in our laboratory; and isoretinenes *a* and *b*, prepared and made available to us by the Organic Research Laboratory of Distillation Products Industries of Rochester, New York (Fig. 23).[9, 50, 51a]

On incubation with opsin, all-*trans* retinene and neoretinene *a* are inactive. Neoretinene *b* yields a rapid synthesis of rhodopsin; the product is indistinguishable from rhodopsin extracted from the retina. Isoretinene *a* yields about the same amount of light-sensitive pigment, and about as quickly; but the spectrum of the pigment is displaced about 13 mμ below that of rhodopsin, λ_{max} falling at about 487 mμ. We have suggested that

this be called isorhodopsin; there is as yet no evidence that it occurs in the living retina. Isoretinene *b* itself appears to be inactive; but on long incubation, or on very short exposure to light, it isomerizes to isoretinene *a*, which yields isorhodopsin (Fig. 24).

It will be noted that we have mentioned five crystalline isomers of retinene, though present theory limits the number of *cis-trans* isomers to

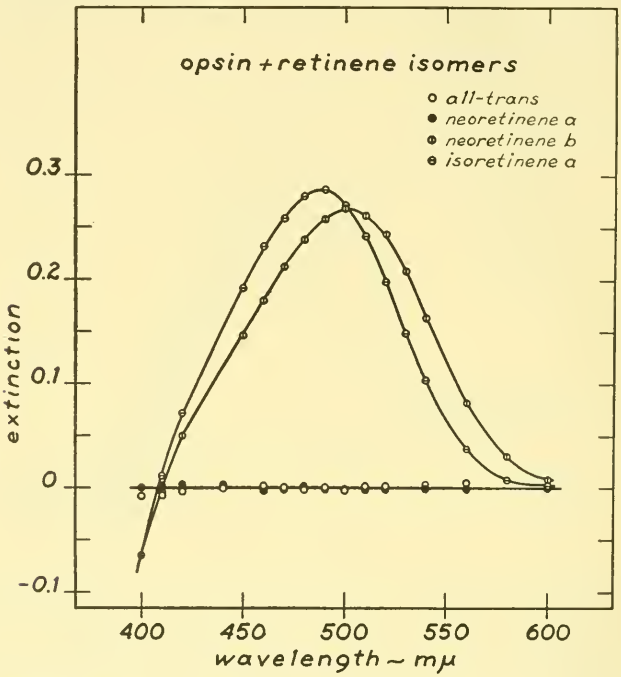


FIG. 24. The synthesis of rhodopsin and isorhodopsin from isomers of retinene. Difference spectra of the products formed when single stereoisomers of retinene are incubated in the dark with cattle opsin. All-*trans* retinene and neoretinene *a* yield no light-sensitive pigment; neoretinene *b* yields rhodopsin (λ_{max} 500 mμ) indistinguishable from that extracted from the dark-adapted retina; and isoretinene *a* yields isorhodopsin (λ_{max} 487 mμ). Isoretinene *b* itself appears to be inactive but isomerizes preferentially to isoretinene *a*, which yields isorhodopsin. (From R. Hubbard and G. Wald.[51a]

four. We have examined the properties of the retinene isomers with particular care, with the thought that one or more of them might involve some type of structural modification other than *cis-trans* isomerism. It has turned out that all five isomers are interconvertible on gentle treatment. All of them, on simple exposure to light, isomerize to what appears to be the same equilibrium mixture of isomers. All of them also yield an identical product in the reaction with antimony chloride, and in the same (molar) amount.

If it were not for the discrepancy with theory, there would be no reason to doubt that all five substances are *cis-trans* isomers of one another. As it is, we believe all of them probably to be *cis-trans* isomers; yet we shall defer coming to a final conclusion until further studies have been completed.[9]

We have shown that the immediate precursor of rhodopsin is the *cis* isomer of retinene called neoretinene *b*. Which isomer of retinene emerges when rhodopsin is bleached? If it were neoretinene *b*, rhodopsin would bleach reversibly; yet it has been clear since the early experiments of Kühne that there is little regeneration of rhodopsin from the products of its bleaching.

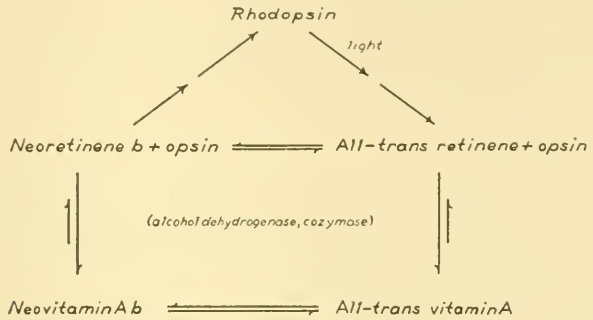


FIG. 25. Geometrical isomers of retinene and vitamin A in the rhodopsin cycle. Retinene enters the synthesis of rhodopsin as neoretinene *b* and emerges from its bleaching all-*trans*. This must be isomerized to neoretinene *b* before it can regenerate rhodopsin. Alternatively it is reduced to all-*trans* vitamin A, which must be isomerized to, or exchanged for, neovitamin A*b* before it can engage again in rhodopsin synthesis. Some isomerization occurs in the eye tissues, but much of the cycle involves also the discard of all-*trans* vitamin A into the blood circulation, and the selection from it of new supplies of neovitamin A*b*. (From R. Hubbard and G. Wald.[51a])

The retinene formed when rhodopsin bleaches is in fact the inactive, all-*trans* isomer. That is, retinene enters rhodopsin as one isomer, and emerges as another. This must be isomerized to neoretinene *b* before it can re-enter the synthesis of rhodopsin. A cycle of stereoisomerization of retinene, or of the corresponding vitamin A, is therefore an intrinsic component of the rhodopsin system.[51, 51a]

The system may now be formulated as in Fig. 25. The bleaching of rhodopsin yields all-*trans* retinene. In the retina this can be isomerized in part by light, in part apparently also by enzymes present in the eye tissues, to neoretinene *b*, which reverts to rhodopsin. Some retinene, before it has isomerized, is reduced by the alcohol dehydrogenase system to all-*trans* vitamin A. This in turn must be isomerized to neovitamin A*b* before it can re-enter the rhodopsin system. Not all of this need occur in the eye itself. There is good evidence that during a long exposure of the eye to

light a large part of the vitamin A of the retina escapes into the circulation.[52] We may assume that during visual activity the retina continuously voids all-*trans* vitamin A into the blood stream and continuously selects neovitamin A*b* out of the circulation by binding it in rhodopsin. These exchanges keep the visual processes in intimate connection with the metabolism of vitamin A throughout the body and with an external factor, its supply in the diet.

We have developed in some detail the biochemistry of the rhodopsin system. The iodopsin system, recently analyzed in the chicken retina, has been found to have precisely the same form.[5] The steroisomers of vitamin A and retinene which take part in the iodopsin system are precisely the same as those in the rhodopsin cycle. Only the protein is different. One has only to replace rhodopsin by iodopsin and to substitute photopsin for opsin—or scotopsin—in the diagram of Fig. 25 to have it represent the iodopsin system.

G. THE GENERAL METABOLISM OF VITAMIN A

I should like now to consider the general metabolism of vitamin A, emphasizing particularly those aspects of its metabolism into which the visual processes yield some insight.

It has recently been shown that retinene, fed to rats by mouth or injected subcutaneously or intraperitoneally, is reduced to vitamin A as it is absorbed.[53] The gut wall and the subcutaneous tissues apparently can accomplish this reduction directly; the site of reduction of intraperitoneal retinene is not known.

If the reduction of retinene involved a specific enzyme, the occurrence of this process in the tissues mentioned might be taken as evidence that they encounter retinene in the course of their ordinary metabolism. This would be of some interest, since retinene has never been found outside the retina. We have seen, however, that retinene is reduced by the highly unspecific and widespread enzyme, alcohol dehydrogenase, whose presence in the tissues is sufficiently explained by their exposure to a variety of metabolic aldehydes. The fact that a tissue can reduce retinene, therefore, is no argument that it ever normally encounters this substance.

It was nevertheless suggested some time ago by Glover *et al.*[53] that retinene is an intermediate in the oxidation of β-carotene to vitamin A. In the past few years it has repeatedly been shown that laboratory procedures for oxidizing β-carotene yield retinene among the products. Hunter and Williams[54] obtained very small yields of retinene (0.4 to 0.5 %) on oxidizing

[52] G. Wald, *J. Gen. Physiol.* **19**, 781 (1935–1936).
[53] J. Glover, T. W. Goodwin, and R. A. Morton, *Biochem. J.* **43**, 109, (1948).
[54] R. F. Hunter and N. E. Williams, *J. Chem. Soc.* **1945**, 554.

β-carotene with hydrogen peroxide in chloroform-acetic acid. More recently Wendler *et al.*[55] obtained yields of retinene as high as 30% on oxidizing β-carotene with hydrogen peroxide-osmium tetroxide.

It is particularly interesting that Meunier *et al.*[56] have reported obtaining retinene in about 60% yield by oxidizing β-carotene on solid manganese dioxide. This is essentially the procedure described earlier for oxidizing vitamin A to retinene.[7, 15] I have pointed out that such "chromatographic oxidations," in which a solid acts at once as adsorbent and reagent, may mimic on occasion the specificity and directedness of an enzymatic reaction. Meunier's observations have not yet been confirmed; and it is already clear from trials in a number of laboratories, including our own, that success in this procedure depends upon conditions which are not yet wholly specified, and which we have not yet been able to reproduce.

The general trend of all these experiments, however, is to encourage the hope that an enzyme system may be found in the tissues which oxidizes β-carotene to retinene. Should such an enzyme—a carotene oxidase—exist, then together with alcohol dehydrogenase to reduce retinene this system would accomplish the complete conversion of β-carotene to vitamin A.

This thought introduces a second consideration. How is such a substance as β-carotene, a hydrocarbon insoluble in aqueous solutions, brought together with enzymes? How indeed is it transported to the blood and lymph, and so distributed to the tissues? The same problems of course exist for retinene and vitamin A.

The answer to the latter question is that, in the blood, carotenoids, probably including vitamin A, are bound to plasma proteins. Palmer and Eckles[57] first showed that in cattle serum β-carotene is bound in a complex with protein which they called "caroto-albumin." In the blood serum of the chicken we found xanthophyll in a similar state.[58]

It seems possible that the existence in the plasma of proteins capable of forming such complexes with carotenoids may be the condition which determines whether or not they are absorbed from the intestine. Zechmeister[59] has stressed the distinction between such "carotene animals" as the horse and cow, which absorb and store almost exclusively the carotenoid hydrocarbons, and "xanthophyll animals," such as the chicken, which store almost exclusively hydroxycarotenoids. Still other forms, such as man and the frog, seem to absorb all types of carotenoids; and such "white fat" animals as the pig and rabbit store almost no plant carotenoids at all.

[55] N. L. Wendler, C. Rosenblum, and M. Tishler. *J. Am. Chem. Soc.* **72**, 234 (1950).
[56] P. Meunier, J. Jouanneteau, and G. Zwingelstein, *Compt. rend.* **231**, 1170 (1950); P. Meunier, *Rev. Intern. Vitaminologie* **23**, 21 (1951).
[57] L. S. Palmer and C. H. Eckles, *J. Biol. Chem.* **17**, 223 (1914).
[58] G. Wald and H. Zussman, *J. Biol. Chem.* **122**, 449 (1938).
[59] L. Zechmeister, *Ergeb. Physiol. biol. Chem. u. exptl. Pharmakol.* **39**, 117 (1937).

Possibly the high selectivity in carotenoid absorption exhibited by some animals and the catholicity by others may have its source in the existence in the blood plasma of more or less specific proteins, which permit the absorption of those carotenoids with which they can combine, and exclude all others from entering the system.

In any case, one can be sure that carotenoids are regularly brought into water solution in the blood by binding to protein. We have shown also that retinene is brought into solution in tissue extracts by a similar process,[27] and Ames and Harris[60] have demonstrated the solubilization of vitamin E by proteins. This appears to be a very general process for carrying fat-soluble substances into water solution in the body fluids and tissues.[61] It may represent the active or "mobilized" form of the carotenoids, as opposed to simple solution in fat globules, the form in which they are stored. It would then be in combination with proteins that carotenoids are made accessible to enzymes; and on occasion the enzyme protein itself would bind them.

This is not even now an empty hypothesis. Retinene has been shown to combine spontaneously with a variety of proteins,[62] and we have direct evidence of its combination in the retina and in tissue extracts with alcohol dehydrogenase and with opsin.[27] Vitamin A also, since it is acted upon by alcohol dehydrogenase, must combine with this enzyme.

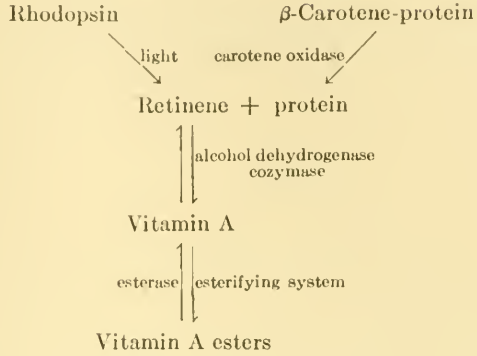
Another process needs to be considered which has been referred to above only in passing. This is the esterification of vitamin A. The vitamin A of the blood plasma is predominantly the free alcohol, whereas that of the liver is overwhelmingly esterified.[63] Glover *et al.*[53] found evidence of an active esterification of vitamin A in the intestinal wall and in subcutaneous tissues. It can be added that vitamin A seems to be esterified rapidly in the eye tissues. We have found in partition experiments with extracts from frog and cattle eyes that the vitamin A of both the retina and the pigment epithelium is predominantly in the ester form.

All these processes taken together compose the following view of vitamin A metabolism. β-Carotene, perhaps in a water-soluble complex with protein, is oxidized by carotene oxidase to retinene. This is reduced by the alcohol dehydrogenase system to vitamin A. Some of the vitamin A is converted in the tissues to esters, which are in turn hydrolyzed to the free alcohol for transport and to perform certain metabolic functions.

The curious thing about this view of events is that it brings the general metabolism of vitamin A into such close relation with the visual processes. In both cases a carotenoid protein is degraded over retinene to vitamin A,

[60] S. R. Ames and P. L. Harris, *Intern. Rev. Vitamin Research* **22**, 26 (1950).
[61] B. D. Davis, *Am. Scientist* **34**, 611 (1946).
[62] S. Ball, F. D. Collins, R. A. Morton, and A. L. Stubbs, *Nature* **161**, 424 (1948).
[63] E. LeB. Gray, K. C. D. Hickman, and E. F. Brown, *J. Nutrition* **19**, 39 (1940); H. M. Kascher and J. G. Baxter, *Ind. Eng. Chem. Anal. Ed.* **17**, 499 (1945).

and this is then esterified. The parallelism between both types of system is apparent in such a diagram as follows:



At present the principal usefulness of such a diagram is to pose a series of problems. What is the nature of the combination of β-carotene—a hydrocarbon bearing no obviously active groups—with protein? Is it true that such protein complexes represent the metabolic form of carotene, and perhaps also of vitamin A? Does there exist in the tissues a "carotene oxidase" that acts as postulated here? What is the enzymatic mechanism which esterifies vitamin A? And so on.

If all these matters were settled, they would dispose only of the vegetative aspects of vitamin A metabolism. They would leave untouched the much more interesting questions involving the utilization of vitamin A in the tissues. Perhaps here also something can be learned from the utilization of vitamin A in the retina. The widespread distribution of the alcohol dehydrogenase system makes it possible for many tissues to convert vitamin A to retinene, particularly in situations in which some receptor molecule is available to condense with and remove retinene from the system. The particular gain in oxidizing vitamin A to retinene as a first step in its utilization is that retinene is an enormously more active molecule. It condenses spontaneously with amino and sulfhydryl groups on proteins and other types of molecules, undergoes addition reactions of various kinds, and exhibits in general the wide variety of reactions that go with the carbonyl group. It is possible that in the synthesis of rhodopsin we have a model for a general class of reactions in which vitamin A, through intermediate conversion to retinene, is attached to other molecules to form the complexes upon which its general cellular activities depend.

In all these reactions the stereoisomerism of vitamin A, its derivatives, and its carotenoid precursors must play a part. As already noted, changes in *cis-trans* configuration affect the *shape* of the molecule; and for reactions with enzymes and other proteins shape is everything. In addition to the

large stereoisomeric effects already demonstrated in visual systems, we may expect to find some degree of stereochemical specificity in all the reactions with proteins in which carotenoids participate.

Since carotenoids isomerize within the body, such effects should be much more limited in the whole animal than in isolated enzyme systems. Nevertheless, considerable differences in the over-all effectiveness of various provitamins A fed to rats have been demonstrated in the laboratories of Zechmeister and Deuel.[64] Stereoisomers of a single carotenoid (e.g., α-carotene) were found to vary in biological activity by as much as 400%. In general, the all-*trans* isomer was most active; though pro-γ-carotene, a poly-*cis* isomer, is about as effective when fed to rats as all-*trans* γ-carotene.

There is good evidence that vitamin A itself stereoisomerizes in the body. It has been reported that neovitamin A*a*[51] and the 3-*cis* retinene synthesized by Graham *et al.*[65] yield bioassays comparable with all-*trans* vitamin A in growth tests in the rat. It has been shown also that, after rats are fed neovitamin A*a*, characteristic mixtures of all-*trans* and *cis* forms of vitamin A are deposited in the liver.[51] The rate at which vitamin A stereoisomerizes in the body is not known, but it seems at least to keep pace with such long-term processes as growth. Even here, however, on more careful examination, differences have emerged. On reviewing the data which had accumulated in the Distillation Products laboratory over a period of seven years, Harris *et al.*[66] concluded that neovitamin A*a* is only 80.7% as effective as the all-*trans* isomer in stimulating growth in rats, and 71.5% as effective in causing the deposition of vitamin A in the liver.

The causes of such over-all differences in biological effectiveness are not yet known. They must principally involve early stages in carotenoid metabolism—differences in stability in the gastrointestinal tract, ease of absorption and transport in the blood, and the first reactions in which these substances take part in the tissues. After a fairly short sojourn in the body, it may be expected that all initial differences in *cis-trans* configuration are obliterated by stereoisomerization.

One of the general methods for stereoisomerizing carotenoids is to warm them. At about 60°, many carotenoids form an equilibrium mixture of isomers within 1 to 2 hours.[49] What takes 1 to 2 hours at 60° might take a day at mammalian body temperature. Perhaps this is all that is involved in the stereoisomerization of carotenoids *in vivo*. Yet, as already mentioned, there is evidence that in the eye the stereoisomerization of vitamin A or retinene is catalyzed by an enzymatic reaction; and it is possible that elsewhere in the body enzymes also have a part in stereoisomerizing the carotenoids.

[64] See the review by L. Zechmeister, *Vitamins and Hormones* **7**, 57 (1949).
[65] W. Graham, D. A. Van Dorp, and J. F. Arens, *Rec. trav. chim.* **68**, 609 (1949).
[66] P. L. Harris, S. R. Ames, and J. H. Brinkman, *J. Am. Chem. Soc.* **73**, 1252 (1951).

These are some of the problems that lie immediately ahead. They scarcely touch as yet upon the most important problem of all—the general role of vitamin A in cellular metabolism. Yet the beginning that has been made with vitamin A metabolism in the study of visual systems seems now to lead in several directions beyond the retina; and it offers some promise of bringing us to this issue also.

IV. Determination

H. H. INHOFFEN and H. POMMER

A. PHYSICAL AND CHEMICAL METHODS

1. General Considerations

A clear distinction must be drawn between determinations on vegetable materials which contain only provitamins A, and determinations on animal materials where both vitamin A and the provitamins are analyzed. A series of color reactions is in use to demonstrate the presence of vitamin A as well as of the provitamins; some of them are also practicable for quantitative estimations. The best-known and most widely used reagent is the solution of antimony trichloride in pure chloroform. This Carr-Price reaction[1] is very sensitive and gives a blue color with provitamins A and vitamins A. But it must be borne in mind that all carotenoids and some synthetic polyene compounds show the same color reaction.[1a] Thus, the appearance of a blue color cannot be regarded always as a proof, and it is therefore necessary to determine at least the maximum of the light absorption of the blue color which for vitamin A_1 is near 617 mμ, for vitamin A_2 near 693 mμ, and for β-carotene near 590 mμ.[2] Recently, F. D. Collins[2a] has published that for β-carotene another maximum of absorption lies near 1020 mμ. As this blue color follows the Lambert-Beer law, it can also be used for the determination of the quantitative ratios. As is well known, all spectrophotometric methods of determination are based on this law. Its equation is

$$\log \frac{I_0}{I_d} = kcd = E$$

in which I_0 represents the intensity of the incident light, I_d is the intensity of the emerging light (monochromatic), k is the extinction coefficient per

[1] F. H. Carr and E. A. Price, *Biochem. J.* **20**, 497 (1926).
[1a] P. Karrer and E. Jucker, Carotinoide. Verlag Birkhäuser, Basel, 1948.
[2] R. A. Morton, *J. Pharm. and Pharmacol.* **2**, 129 (1950).
[2a] F. D. Collins, *Nature* **165**, 818 (1950).

concentration unit, c is the concentration in grams per liter, and d is the thickness of the layer in centimeters.

The extinction coefficient k is often given as the molar extinction coefficient ϵ (gram-moles per liter).

$$\epsilon = \frac{E \cdot M}{c \cdot d} = k \cdot M \qquad (M = \text{molecular weight})$$

In vitamin chemistry the term $E^{1\%}_{1\,\text{cm.}}$ is commonly used, according to the formula

$$c(\%) = \frac{E}{E^{1\%}_{1\,\text{cm.}} \cdot d(\text{cm.})}$$

which immediately gives the concentration c in per cent. In practice, it generally refers to the material being examined; e.g., fish liver oils, which show at 328 mμ an $E^{1\%}_{1\,\text{cm.}}$ of 17.5, contain 1 % vitamin A. Furthermore, the color reaction with glycerol dichlorohydrin is important for it gives a stable red color.[2b] With regard to the quantitative determination, this color reaction offers some advantages in comparison to the Carr-Price reaction. The color is stable for 2 to 10 minutes, and the reagent is considerably less affected by moisture than antimony trichloride in chloroform. The maximum of absorption for the determination of vitamin A alcohol with this reagent lies near 550 mμ, $E^{1\%}_{1\,\text{cm.}} = 1150$ to 1250. However, this color reaction is not yet sufficiently tested to warrant general acceptance for quantitative purposes, but its importance may increase.[3] In principal, the same methods serve for the determination of carotene and vitamin A after appropriate preparation. Thus, measurement of the quantitative extinction of the light absorption maxima and comparison with a standard curve represents in both cases the most precise method. Beside this method the old colorimetric or photometric procedure is still frequently used for the determination of β-carotene. Standard β-carotene, azobenzene,[4] or potassium bichromate solutions[5] are used as comparison solutions for this procedure (see below). Besides, as mentioned above, the blue color with antimony trichloride in chloroform or the red color with glycerol dichlorohydrin is employed for the quantitative determination, especially for vitamin A. Here also the measurement of the intensity is done with a colorimeter, with a photometer, or better with a spectrophotometer.

The determination with the Carr-Price reaction gives rise to several

[2b] A. E. Sobel and H. Werbin, *J. Biol. Chem.* **159**, 681 (1945); *Anal. Chem.* **19**, 107 (1947).

[3] P. B. Müller, *Helv. Chim. Acta* **30**, 1172, 1188 (1947).

[4] R. Kuhn and H. Brockmann, *Hoppe-Seyler's Z. physiol. Chem.* **206**, 41 (1932).

[5] A. Krogis, *Biochem. Z.* **287**, 266 (1936).

sources of error.[6, 7] Even small amounts of moisture cause considerable error, and furthermore the presence of unsaturated fats or indole derivatives influence the development of the blue color. As the maximum of intensity of the blue color is reached after 5 to 10 seconds, and then the "blue value" drops quite rapidly, the measurement must be carried out during this short period. This involves uncertainties and requires a greater number of control measurements. For this reason the international arrangement gives chief attention to the quantitative measurement of the ultraviolet absorption maximum of vitamin A. But still the U. S. Pharmacopeia demands the control of this value by the Carr-Price reaction,[8] which delivers useful results if expertly performed. The spectrophotometric measurement of the maxima of light absorption can be carried out with any spectrophotometer or suitable spectral apparatus, but nowadays the preference is generally for the much easier and more precise measurement by a spectrophotometer, e.g., of the Beckman-DU type, to the old method by photography of the spectrum and evaluation by quantitative photometry. Hilger's Vitameter[9] is also used for the determination of the vitamin A content. Here, too, a special vitamin A standard preparation is useful, because it permits comparative measurement with the individual instrument and thereby enables the analyst to compensate for faults of the apparatus.

The influence of the solvent on the spectrum of vitamin A and of β-carotene has been investigated by several workers.[10-12] Nowadays, the quantitative measurement of the blue color of the Carr-Price reaction may appropriately be carried out with a spectrophotometer, but in many countries colorimetric measurement by the Lovibond Tintometer[13] is still in use. The reaction solution is compared with glass standards having different shades of blue color. Often the measurement is done by a Zeiss-Pulfrich Stufen-Photometer with filter S61.[14, 15] Generally, the Carr-Price determination of vitamin A in fish liver oils, butter, and milk can be made only on the unsaponifiable fraction, because the raw material contains substances which interfere with the color reaction. (On the preparation and conservation of the Carr-Price reagent, see Müller.[16])

[6] M. J. Caldwell and D. B. Parrish, *J. Biol. Chem.* **158**, 181 (1945).
[7] M. J. Caldwell and J. S. Hughes, *J. Biol. Chem.* **166**, 565 (1946); **170**, 97 (1947).
[8] U. S. Pharmacopeia, 14th revision, pp. 784, 792, 1950.
[9] R. J. Taylor, *Analyst* **67**, 248 (1942).
[10] A. E. Gillam and M. S. El Ridi, *Biochem. J.* **32**, 1496 (1938).
[11] F. P. Zscheile and R. L. Henry, *Ind. Eng. Chem. Anal. Ed.* **14**, 422 (1942).
[12] P. Karrer and E. Jucker, Carotinoide. Verlag Birkhäuser, Basel, 1948.
[13] O. Rosenheim and E. Schuster, *Biochem. J.* **21**, 1329 (1927).
[14] L. Fuchs and E. Soos, *Vitamine u. Hormone* **4**, 155 (1943).
[15] M. Lodi, *Vitamine u. Hormone* **4**, 404 (1943).
[16] P. B. Müller, *Mitt. Gebiete Lebensm. u. Hyg.* **40**, 359 (1949); see also ref. 3.

2. Determination of Vitamins A

Experiments on animals are costly and allow serial experiments only in special cases. Therefore, analysts have always wanted to go from biological assay to chemical analysis which is quicker and easier to perform. After statistically significant data on pure and crystalline compounds were obtained by biological assay of vitamin A, the analytical problem shifted to chemical methods.

As was proved by careful comparison between the biological and spectrophotometric vitamin A determinations, especially with whale liver oils, over- and undervaluing was observed when the same conversion factor was used.[2] This was because the oils contain substances which also absorb near the ultraviolet maximum of vitamin A and cause a shift of the maximum and also affect the extinction coefficient. This phenomenon is termed "irrelevant absorption." Morton and Stubbs[17] elaborated a method which eliminates the irrelevant absorption. A simplification of this correction method is given by Oser.[18] Whether correction is necessary can be answered only after a careful spectrophotometric survey of the extinction curve. Vitamin A ester concentrates and most good molecular distillates do not need any correction. Cod liver oils nearly always require correction, if the determination is made on the unsaponified oil. Therefore, if possible, in this case the determination is carried out on the unsaponifiable fraction. Whale liver oils or oils stored for a long time nearly always require correction, even if the determination is made with the unsaponifiable material. The technical details of the correction according to Morton and Stubbs may be found in the original papers.[2, 17]

In cases of strong irrelevancy it is advisable to submit the samples to chromatographic purification and to isolate the vitamin A in as pure form as possible. As the latest methods of determination[19-21] based on this principle show, a spectrophotometer gives an absorption curve which is comparable with that of the standard and which allows unhesitating application of the conversion factor of 1900. Considering the great amount of work dealing with the estimation of vitamin A, it is not possible to give more than a few examples. Further details are given in the special literature on this analysis.[22-24] The vitamin A determination in fish liver oils is described

[17] R. A. Morton and A. L. Stubbs, *Analyst* **71**, 356 (1946); *Biochem. J.* **41**, 525 (1947); **42**, 195 (1948).

[18] B. L. Oser, *Anal. Chem.* **21**, 529 (1949).

[19] N. T. Gridgeman, G. P. Gibson, and J. P. Savage, *Analyst* **73**, 662 (1948).

[20] R. K. Barua and R. A. Morton, *Biochem. J.* **45**, 308 (1948).

[21] W. Hjarde, *Acta Chem. Scand.* **4**, 628 (1950).

[22] F. Gstirner, Chemisch-physikalische Vitamin-Bestimmungs-Methoden, 4th ed. Ferdinand Enke, Stuttgart, 1951.

[23] W. J. Dann, *Biol. Symposia* **12**, 13 (1947).

by Morton and Stubbs.[17] The estimation in foodstuffs is in most cases possible only after saponification and chromatography. Thus, a method for determination in dried eggs, which describes the separation of vitamin A from xanthophylls in egg yolk, was worked out by Thompson *et al.*[25] and by Schrenk *et al.*[26] Mann,[27] as well as Raynes and McLellan,[28] give a description for the determination of the vitamin A content of vitaminized cocoa and chocolate goods. The vitamin A and carotene content of milk, butter, and margarine has been discussed in many investigations.[29-43] Universally applicable prescriptions for the determination in fish liver oils, margarine, butter, milk, and other foods have been published by Gridgeman and his collaborators[19] and by Hjarde.[21] Both use the chromatographic adsorption method for the purification of the vitamin A.

As mentioned before, fish liver oils often show irrelevant absorption. Therefore, a series of investigations was carried out to make the determination more specific, by testing the material in the usual way, then destroying the vitamin A through oxidation, and again measuring spectrophotometrically. From the difference, the vitamin A content may be calculated.[44, 45] But these methods cannot be credited with special importance. Somewhat

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[24] H. R. Rosenberg, Chemistry and Physiology of the Vitamins. Interscience Publishers, New York, 1945.

[25] C. R. Thompson, M. E. Ewan, S. M. Hauge, B. B. Bohren, and F. W. Quackenbush, *Ind. Eng. Chem. Anal. Ed.* **18**, 113 (1946).

[26] W. G. Schrenk, D. S. Chapin, and R. M. Conrad, *Ind. Eng. Chem. Anal. Ed.* **16**, 632 (1944).

[27] T. B. Mann, *Analyst* **68**, 233 (1943).

[28] E. G. Raynes and B. G. McLellan, *Analyst* **68**, 109 (1943).

[29] P. D. Boyer, R. Spitzer, C. Jensen, and P. H. Phillips, *Ind. Eng. Chem. Anal. Ed.* **16**, 101 (1944).

[30] J. Effern, *Vorratspflege u. Lebensmittelforsch.* **3**, 53 (1940).

[31] F. R. Olson, D. M. Hegsted, and W. H. Peterson, *J. Dairy Sci.* **22**, 63 (1939).

[32] M. Hochberg, *J. Dairy Sci.* **31**, 315 (1948).

[33] L. Skurnik and M. Hellen, *Z. Vitaminforsch.* **15**, 52 (1944).

[34] F. P. Zscheile, R. L. Henry, J. E. White, Jr., H. A. Nash, C. L. Shrewsbury, and S. M. Hauge, *Ind. Eng. Chem. Anal. Ed.* **16**, 190 (1944).

[35] F. P. Zscheile, H. A. Nash, R. L. Henry, and L. F. Green, *Ind. Eng. Chem. Anal. Ed.* **16**, 83 (1944).

[36] W. Wodsak, *Pharmazie* **4**, 370 (1949).

[37] A. E. Sobel and A. A. Rosentag, *Anal. Chem.* **21**, 1540 (1949).

[38] P. B. Müller and H. Moor, *Mitt. Gebiete Lebensm. u. Hyg.* **40**, 358 (1949).

[39] J. B. Wilkie, *J. Assoc. Offic. Agr. Chemists* **30**, 382 (1947).

[40] I. L. Bowen, N. T. Gridgeman, and G. F. Longman, *Analyst* **71**, 20 (1946).

[41] J. R. Edisbury, *Analyst* **65**, 484 (1940).

[42] T. W. Goodwin and R. A. Morton, *Analyst* **71**, 15 (1946).

[43] W. Heimann, *Z. Untersuch. Lebensm.* **85**, 502 (1943).

[44] A. Fujita and T. Sakamoto, *Biochem. Z.* **308**, 344 (1941).

[45] G. T. Jones and R. T. Harris, *Analyst* **68**, 8 (1943).

more important for special cases is the method of Demarest,[46] which is based on selective destruction of vitamin A by means of ultraviolet irradiation.

A paper-chromatographic method for the determination of vitamin A is given by Datta and Overell.[47] The separation of vitamin A, vitamin A esters, and carotene by chromatography on aluminium oxide activated to different degrees was carried out by Müller;[48] this method is generally applicable. Ritsert[49] discussed thoroughly the separation of vitamin A from vitamin A esters by shaking the extract with methanol. In order to isolate neovitamin A one makes use of its slow reaction with maleic acid anhydride, with which vitamin A condenses rapidly.[50]

The amounts of vitamin A_2 occurring in commercial liver oils are so small that one can dispense with their determination. Otherwise, this may be done only spectrophotometrically after separation by chromatography;[51] the same applies to kitols.[51]

3. Determination of Provitamins A

Although the determination of vitamin A seems to be fully worked out, that of the provitamins A is far from satisfactory. A determination of provitamins A means, in practice, the measurement of carotenes. Hitherto, the basic method of carotene determination, as documented by Kuhn and Brockmann's classical procedure, has not undergone important changes, but many new findings in recent years have led to refinements. One serious problem is the quantitative extraction of the carotenes from vegetable material. Kuhn and Brockmann[4] determined the total content of carotene without separation of the isomers. But lately such a separation has become a rule, for it is realized that α-carotene shows only 50 %, and γ-carotene only 25 %, of the biological activity of β-carotene.[52-54] Recently, this problem became more complicated by the results of Fraps, Zechmeister, Zscheile, and their collaborators,[55] who found the stereoisomers of carotene in various

[46] B. Demarest, *Z. Vitaminforsch.* **9**, 20 (1939); A. Chevallier, *ibid.* **7**, 10 (1938).

[47] S. P. Datta and B. G. Overell, *Biochem. J.* **44**, XLIII (1949); H. P. Kaufmann, *Fette u. Seifen* **52**, 331 (1950).

[48] P. B. Müller, *Helv. Chim. Acta* **26**, 1945 (1943); **30**, 1172 (1947).

[49] K. Ritsert, *Vitamine u. Hormone* **3**, 57 (1943).

[50] C. D. Robeson and J. G. Baxter, *J. Am. Chem. Soc.* **69**, 136 (1947).

[51] R. A. Morton and A. Stubbs, *Biochem. J.* **40**, LVIII (1946); R. K. Barua and R. A. Morton, *Biochem. J.* **45**, 308 (1948).

[52] H. J. Deuel, Jr., E. Sumner, C. Johnston, A. Polgár, and L. Zechmeister, *Arch. Biochem.* **6**, 157 (1945).

[53] H. J. Deuel, Jr., C. Johnston, E. Sumner, A. Polgár, W. A. Schroeder, and L. Zechmeister, *Arch. Biochem.* **5**, 365 (1944).

[54] A. R. Kemmerer and G. S. Fraps, *J. Biol. Chem.* **161**, 305 (1945).

[55] B. W. Beadle and F. P. Zscheile, *J. Biol. Chem.* **144**, 21 (1942).

plants. According to the results of these workers, the stereoisomers do not have full biological activity[56, 57] and should therefore be separated for exact values. But in most cases the error arising from failure to separate the stereoisomers is insignificant enough to be neglected. On the whole, it is a recurring question for the analyst as to what degree of exactness is required in each case. In case of clinical determinations, it is practically always sufficient to determine the total content of carotene, especially because the analytic method is not exact, since only very small quantities of material are available.

The preparation of vegetable material for analysis requires considerable skill, and it varies to a certain extent for each plant. It is not possible to transfer the results from one case to another (see also Zscheile and Whitmore[58]). The difficulties begin with the collection of the fresh vegetable material. These samples are suitably and immediately stored in the cold, if possible below 0°; otherwise a loss of carotene may occur. Conditioned in this way the carotene content remains unchanged for a long time. Zscheile and Whitmore[58] found that fresh alfalfa leaves stored for 66 days at −18° had the same carotene content as on the first day. Before extraction the fresh vegetable samples should be treated with boiling water for 5 to 10 minutes in order to destroy the enzymes. According to investigations of H. Süllmann[59-61] a rapid enzymatic destruction takes place in the presence of unsaturated fatty acids. To neutralize the vegetable acids Zscheile[58] adds magnesium carbonate to the extractant. He uses 40% acetone as solvent for the extraction of fresh vegetable material. In other cases methanol, ethanol, petroleum ether, and ether are suitable solvents. Dried material is more difficult to extract than fresh plant tissues. An extended extraction with boiling solvents often leads to a loss of carotene and also isomerization. For the extraction of dried alfalfa Zscheile and Whitmore[58] recommended a mixture of hexane and acetone (7:3). Investigation of the methods for extracting carotene from dried vegetable material must be regarded as not yet concluded.

The analytic separation of the extract is done by chromatography. Here the selection of the adsorption agent depends on the composition of the extract. In use are aluminum oxide,[62] calcium hydroxide,[63] magnesium

[56] H. J. Deuel, Jr., C. Johnston, E. Sumner, A. Polgár, and L. Zechmeister, *Arch. Biochem.* **5**, 107 (1944); **6**, 157 (1945); **7**, 247 (1945).

[57] H. J. Deuel, Jr., C. Hendrick, E. Straub, A. Sandoval, J. H. Pinckard, and L. Zechmeister, *Arch. Biochem.* **14**, 97 (1947).

[58] F. P. Zscheile and R. A. Whitmore, *Anal. Chem.* **19**, 170 (1947).

[59] J. B. Sumner and A. L. Dounce, *Enzymologia* **7**, 130 (1939).

[60] H. Süllmann, *Helv. Chim. Acta* **24**, 646 (1941).

[61] E. V. Hove, *Science* **98**, 433 (1943).

[62] G. Wendland, *Vitamine u. Hormone* **5**, 46 (1944).

[63] A. Fujita, T. Narita, and M. Agisaka, *Biochem. Z.* **308**, 420 (1941).

oxide,[64] magnesium hydroxide,[65] magnesium carbonate,[65] calcium phosphate,[66] silicic acid,[58] zinc carbonate,[67] and bone powder.[68]

It is necessary to test in each case whether the extracted pigments can be directly separated by chromatography or whether a preliminary purification is necessary. Such a purification can be achieved by saponification whereby the chlorophyll is separated and carotenoid esters, (e.g., xanthophyll esters) are saponified. After the unsaponifiable fraction has been isolated it can be suitably partitioned between 95 % methanol and petroleum ether, the chromatographic analysis being carried out with the epiphase (petroleum ether phase). Of the more important carotenoids, carotene and lycopene are to be found in the epiphase (see table in Karrer and Jucker, p. 29 in ref. 12). It should be noticed that cryptoxanthin and rubixanthin will also become epiphasic with 90 % methanol. Instead of methanol, 94 % aqueous diacetone alcohol can be used to advantage.[69] According to Haagen-Smit[70] the separation of xanthophyll can be performed by extraction with 85 % phosphoric acid. As solvent for the chromatographic adsorption *n*-hexane (b.p. 65 to 67°) or pure petroleum ether (optically pure) (b.p. 40 to 60°) is used almost exclusively. Technical details about these procedures are given by Zechmeister[71] and Zscheile[72] (see also Karrer and Jucker[12] and Hesse[67]). However, the quantitative exactness of the chromatographic adsorption analysis is limited. Losses through oxidation as a consequence of an increased reactivity while being adsorbed, through the action of light, and through isomerization and persistent adhesion of the pigment to the adsorbent cannot be completely excluded. These losses increase, the smaller the quantity of the substances being determined. In general, they range from 2 to 5 %. With quantities of 150 to 30 γ they can range from 20 to 50 % (see Gstirner, p. 39 in ref. 22). Hence, for exact values, it is necessary to determine the average losses with known quantities of the carotene and to correct the analytic figures accordingly.

The identification of the isomeric and stereoisomeric carotenes isolated from the chromatogram is done spectroscopically (for the absorption curves

[64] A. R. Kemmerer and G. S. Fraps, *Ind. Eng. Chem. Anal. Ed.* **15**, 714 (1943).
[65] A. R. Kemmerer, *J. Assoc. Offic. Agr. Chemists* **27**, 542 (1944); **28**, 563 (1945); **29**, 18 (1946).
[66] L. A. Moore, *Ind. Eng. Chem. Anal. Ed.* **12**, 726 (1940); **13**, 600 (1941); **14**, 707 (1942).
[67] G. Hesse, Die Adsorptionsmethoden im chemischen Laboratorium. W. de Gruyter, Berlin, 1943.
[68] T. B. Mann, *Analyst* **69**, 34 (1944).
[69] D. M. Hegsted, J. W. Porter, and W. H. Peterson, *Ind. Eng. Chem. Anal. Ed.* **11**, 256 (1939).
[70] A. J. Haagen-Smit, C. E. P. Jeffreys, and J. G. Kirchner, *Ind. Eng. Chem. Anal. Ed.* **15**, 179 (1943).
[71] L. Zechmeister, *Chem. Revs.* **34**, 267 (1944).
[72] F. P. Zscheile, *Arch. Biochem.* **5**, 77, 211 (1944).

see Zechmeister,[71] Zscheile,[72] also Karrer and Jucker[12]). Nowadays the quantitative determination is performed exclusively by spectrophotometry, measuring the molaric extinction coefficient at a maximum of the absorption. For clinical assay and in older works, however, it is frequently carried out either colorimetrically against standard carotene,[74] azobenzene,[4] or potassium bichromate,[5] as well as photometrically. The Zeiss-Pulfrich photometer with filter S43 is appropriate for the last-named method.[63]

From what has been said above, it follows that for the exact investigation of material containing provitamins A a special procedure must be developed in each case; often only a slight modification is needed. Thus the methods quoted here frequently should serve only as guides. From the great number of methods proposed the following will be selected as being either very special or possessing a relatively broad range of application. The procedure of Fujita *et al.*[63] is a direct continuation of the well-known method of Kuhn and Brockmann.[4] As described by the authors, it is not suitable for vegetables rich in fat and wax or for leavened green fodder. Zscheile and Whitmore[58] have elaborated a rather exact method for carotene in fresh and dried alfalfa. The determination of carotene and lycopene in lycopersicon species and strains was carried out by Zscheile and Porter.[73] Further statements concerning the carotene content in vegetable tissues have been made by a number of research workers.[75-82] According to White and Zscheile[83] the distribution of β-carotene, cryptoxanthin, and zeaxanthin between hexane, aqueous methanol, diacetone alcohol, and 2-methylpentanediol-(2,4) makes it possible to separate these pigments. A determination in material rich in fat, e.g., vitaminized chocolate, is given by Vastagh.[84] The same communication contains a prescription for the estimation of the cryptoxanthin content.

4. Determination of Vitamin A and Provitamins A Concurrently

A simultaneous determination of vitamin A and provitamins A becomes necessary in clinical assays and also when butter, milk, eggs, and artificially

[73] F. P. Zscheile and J. W. Porter, *Anal. Chem.* **19**, 47 (1947).
[74] *World Health Organisation Tech. Rept. Ser.* **30**, (1950).
[75] M. E. Wall and E. G. Kelley, *Ind. Eng. Chem. Anal. Ed.* **15**, 18 (1943).
[76] H. L. Mitchell and S. M. Hagne, *J. Biol. Chem.* **164**, 543 (1946).
[77] H. L. Mitchell and H. H. King, *Anal. Chem.* **20**, 637 (1948).
[78] R. E. Silker, W. G. Schrenk, and H. H. King, *Ind. Eng. Chem. Anal. Ed.* **16**, 513 (1944).
[79] W. A. G. Nelson, *Analyst* **72**, 200 (1947).
[80] H. L. Mitchell, *Plant Physiol.* **24**, 323 (1949).
[81] A. Sreenivasan and R. M. Vaidya, *Anal. Chem.* **20**, 720 (1948).
[82] O. Hromatka and R. Kerl, *Monatsh. Chem.* **78**, 139 (1948).
[83] J. W. White and F. P. Zscheile, *J. Am. Chem. Soc.* **64**, 1440 (1942).
[84] G. Vastagh, *Z. Untersuch. Lebensm.* **86**, 393 (1943).

vitaminized foods are examined. The only method which permits concurrent determination is the chromatographic adsorption analysis. However, particularly in clinical investigations, this is usually not practicable because of small quantities of material. Therefore, in the case of serum, the isolation of the vitamin A and the carotenes in pure state is usually avoided. The proper determination is made by means of the blue color with antimony trichloride in pure chloroform. Human serum contains vitamin A and carotenoids with small proportions of carotene, whereas the serum of cattle contains relatively small amounts of vitamin A and is rich in quantities of carotene. As carotenoids also react upon antimony trichloride with a blue color, conditions are complicated and corrections become necessary. Hence it appears that, according to Gillam and Senior[85] and Willstaedt and With,[86] the carotenoids have to be separated by partitioning between petroleum ether and methanol. In the case of human serum, however, a correction is necessary even then because up to 50% of the total carotenoids may be hypophasic. Boyer *et al.*[87] perform the separation by precipitation of the carotene from alcoholic solution by diluting to 50 to 60%; hereby the vitamin A remains dissolved. This method is especially suited for sera rich in carotene. Willstaedt and With[88] worked out a photometric method which uses as a measure for the correction of the vitamin A value the ratio of the extinctions between the measurements with filter S61 (Pulfrich photometer) after adding antimony trichloride and with filter S43 without such addition. Of great importance for the quantitative determination of vitamin A and carotene is the mode of extraction applied, especially for serum. Both components are combined with protein which must be denaturated. Yudkin[89] and Glover *et al.*[90] precipitate with alcohol and extract with petroleum ether (protein precipitation method), whereas Lindquist[91] as well as Nylund and With[92] saponify with alkali and extract afterwards (saponification method). Further works deal with the examination of blood and serum.[93, 94] Veldman[95] reports a microdetermination method. Sobel and

[85] A. E. Gillam and B. J. Senior, *Biochem. J.* **30,** 1249 (1936).

[86] H. Willstaedt and T. K. With, *Hoppe-Seyler's Z. physiol. Chem.* **253,** 133 (1938).

[87] P. D. Boyer, P. H. Phillips, and J. K. Smith, *J. Biol. Chem.* **152,** 445 (1944); *Biochem. J.* **46,** 259 (1950).

[88] H. Willstaedt and T. K. With, *Z. Vitaminforsch.* **9,** 212 (1939); *Biochem. J.* **41,** 97 (1947).

[89] A. M. Yudkin, *Biochem. J.* **35,** 551 (1941).

[90] J. Glover, T. W. Goodwin, and R. A. Morton, *J. Biol. Chem.* **152,** 445 (1944).

[91] T. Lindquist, Dissertation, University of Upsala, 1938; *Acta Med. Scand. Suppl.* **97,** 314 (1938).

[92] C. E. Nylund and T. K. With, *Vitamine u. Hormone* **1,** 354 (1941).

[93] O. A. Bessey, O. H. Lowry, M. J. Brock, and J. A. Lopez, *J. Biol. Chem.* **166,** 177 (1946).

[94] G. H. Wise, F. W. Atkeson, M. J. Caldwell, D. B. Parrish, and J. S. Hughes, *J. Dairy Sci.* **30,** 279 (1947); *J. Animal Sci.* **7,** 70 (1948).

Snow[96] describe a modified procedure with glycerol dichlorohydrin to determine the vitamin A level in blood and serum. The vitamin A and carotene content in feces can be determined by the method of With.[97] The same author discusses in detail investigations on the range of error of the "blue value disturbance" by carotene with the Carr-Price reaction. But one should be reminded that statements about the vitamin A and carotene content of blood and blood serum represent in most cases only approximate values. Only a few of the great number of methods proposed for the examination of tissue, especially liver samples, can be mentioned.[98-100] In most cases they differ only in the preparation of the testing material. The procedure published by Glover, Goodwin, and Morton[101, 102] permits the determination of esterified vitamin A, free vitamin A, and carotene simultaneously. With[103] has worked out a micromethod for the vitamin A determination in liver samples taken at biopsy. The vitamin A and carotene content in milk, butter, and margarine is determined exclusively after saponification.[28] For the examination of eggs a series of methods has been elaborated. The conditions are here more complicated in that the main part of the carotenoids does not consist of the biologically active carotene but of the inactive xanthophyll, zeaxanthin, and others. In such cases the isolation in pure state by chromatographic adsorption is indispensable. Corresponding methods were worked out by Gillam and Heilbron,[104] Thompson and collaborators,[25] Mann,[27] and Schrenk *et al.*[26] Narod and Verhagen[105] have elaborated a procedure for the determination of vitamin A in foodstuffs which have been artificially vitaminized.

B. BIOLOGICAL METHODS FOR THE DETERMINATION OF VITAMIN A ACTIVITY[106-107]

Rats are used nearly exclusively for biological assays. According to Coward,[108] male rats are more sensitive to vitamin A deficiency than are

[95] H. Veldman, *Acta Brevia Neerl. Physiol. Pharmacol. Microbiol.* **11**, 178 (1941).
[96] A. E. Sobel and S. D. Snow, *J. Biol. Chem.* **171**, 617 (1947).
[97] T. K. With, *Z. Vitaminforsch.* **11**, 298 (1941).
[98] H. B. Jensen and T. K. With, *Biochem. J.* **33**, 1771 (1939); *Vitamine u. Hormone* **3**, 254 (1943).
[99] F. Harms, *Vitamine u. Hormone* **2**, 151 (1942).
[100] W. D. Gallup, J. A. Hoefer, *Ind. Eng. Chem. Anal. Ed.* **18**, 288 (1946).
[101] J. Glover, T. W. Goodwin, and R. A. Morton, *Biochem. J.* **41**, 94, 97 (1947).
[102] T. W. Goodwin and R. A. Morton, *Analyst* **71**, 15 (1946).
[103] T. K. With, *Biochem. J.* **40**, 249 (1946).
[104] A. E. Gillam and J. M. Heilbron, *Biochem. J.* **29**, 1064 (1935).
[105] M. Narod and D. Verhagen, *Anal. Chem.* **20**, 627 (1948).
[106] N. T. Gridgeman, The Estimation of Vitamin A. Lever Brothers and Uni Lever Ltd., London, 1944.
[107] E. M. Nelson and J. B. de Witt, *Biol. Symposia* **12**, 1 (1947).
[108] K. H. Coward, *Brit. Med. J.* **I**, 435 (1942).

female animals; therefore equal numbers of males and females are used for the experiment. The age of the experimental animals should be 20 to 22 days, and their weight should not exceed 30 to 40 g.[109]

Vitamin A-deficiency diets are given by Bacharach and his collaborators[110] and others.[111, 112] Jürgens[109] recommends:

Casein, vitamin A-free	18.0%
Rice starch	54.0%
Cocoa fat[a]	13.0%
Salt mixture[b]	5.0%
Dried yeast, light-colored, not irradiated	10.0%
	100.0%

[a] The vitamin A was destroyed by heating up to 90° and simultaneous passing air through for 8 hours.
[b] Sodium chloride, 5.0%; calcium lactate, 35.0%; calcium phosphate, 15.0%; ferrous citrate, 3.21%; potassium iodide, 0.09%; copper sulfate, 0.03%; magnesium sulfate, 5.5%; potassium phosphate, 26.53%; sodium phosphate, 9.6%; zinc carbonate, 0.02%; manganese sulfate, 0.02%; sodium fluoride, 0.003%.

In recent investigations attention was drawn to the synergism existing between vitamin A and tocopherol. Thus, through the presence of tocopherol a favorable influence is exerted on the storage of vitamin A and carotene.[113-115] With the storage test the saving of vitamin A has to be taken into consideration, conditional to this special test (see below).

For biological evaluation the growth test is preferred. Generally, animals fed on a vitamin A-free diet are fit for the test after 6 to 7 weeks, as evidenced by the cessation of growth and the appearance of the first symptoms of xerophthalmia. In all experiments the parallel testing of a vitamin A standard preparation is essential. During the testing period the vitamin A standard as well as the sample to be examined are given daily, and the growth of the animals—that is, their gain in weight—is observed for at least 4 weeks. Only animals of the same sex are comparable. Gridgeman[106] has worked out a method for the evaluation (see also Irwin[116]). The details may be found in the original literature.[106]

Lately, the storage test of Guggenheim and Koch[117] is being recommended as more valid than the growth test. This test requires only one-third to one-fourth the time needed for the growth test. Rats 18 to 22 days old and

[109] R. Jürgens, *Arzneimittel-Forsch.* **1**, 119 (1951).
[110] A. L. Bacharach, E. M. Cruickshank, K. M. Henry, S. K. Kon, J. A. Lovern, T. Moore, and R. A. Morton, *Brit. Med. J.* **1942, II**, 691.
[111] C. A. Cabell and N. R. Ellis, *Proc. Soc. Exptl. Biol. Med.* **65**, 117 (1947).
[112] L. Randoin and J. Causeret, *Bull. soc. sci. hyg. aliment.* **35**, 14 (1947).
[113] K. C. D. Hickmann, *Ann. Rev. Biochem.* **12**, 356 (1943).
[114] K. C. D. Hickmann, M. W. Kaley, and P. L. Harris, *J. Biol. Chem.* **152**, 313 (1944).
[115] T. Moore, *Biochem. J.* **34**, 1321 (1940).
[116] J. O. Irwin, *J. Hyg.* **43**, 291 (1944).
[117] K. Guggenheim and W. Koch, *Biochem. J.* **38**, 256 (1944).

weighing 40 to 50 g. are assembled in groups of twelve and fed a vitamin A-free diet for 6 days. The stored vitamin A reserve is said to be used up in that time. Then for 2 days, the animals of one group are fed the sample to be tested and another group is fed a vitamin A standard preparation. After another 2 days the animals are killed and the vitamin A content of the liver determined.[118]

In 1940 Irving and Richards[119] described a biological method of assay which uses in a prophylactic test the histological changes of the medulla, caused by vitamin A deficiency. This method is rather sensitive and is regarded to be very reliable by Goetzee,[120] so long as the sex is taken into consideration. Male rats require approximately 1.5 times as much vitamin A as females of the same age in order to overcome the symptoms of deficiency.

V. Standardization of Activity

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Improvements of the biological, chemical, and physical assay methods, and the abundance of knowledge involved, brought about a revision of the international vitamin A standard[1] (for information on the development of the vitamin A standards, see Morton[2]). The conference of the expert Committee on Biological Standardization, World Health Organisation, in 1949 in London, recommended new international standards.[3] According to the new proposals, the old β-carotene standard[1] was retained as reference standard for provitamin A but not for vitamin A. Thus, one international unit (I.U.) of provitamin A is equivalent to 0.6 γ of β-carotene. A new standard was fixed for vitamin A. The test substance selected was vitamin A acetate, which is easily obtained in a crystalline and pure state and shows sufficient stability on storage in the cold and in vacuum.[4] An international unit of vitamin A is equivalent to 0.344 γ of vitamin A_1 acetate (0.300 γ of vitamin A alcohol). This standard was fixed after careful statistical evalua-

[118] W. D. Gallup and J. A. Hoefer, *Ind. Eng. Chem. Anal. Ed.* **18**, 288 (1946).

[119] J. T. Irving and M. B. Richards, *Biochem. J.* **34**, 198 (1940).

[120] W. H. R. Goetzee, *Biochem. J.* **45**, 628 (1949).

[1] Report of the Conference on Vitamin Standards, Geneva, 1931; League of Nations Publication, C. H. 1055 (1); Second Conference on Vitamin Standardization, *Quart. Bull. Health Organisation League Nations* **3**, 428 (1934).

[2] R. A. Morton, *J. Pharm. and Pharmacol.* **2**, 129 (1950).

[3] *World Health Organization Tech. Rept. Ser.* **30**, 1–7 (1950).

[4] N. B. Guerrant, M. E. Chilcote, H. A. Ellenberger, and R. A. Dutcher, *Anal. Chem.* **20**, 465 (1948).

tion of the biological comparison of β-carotene and vitamin A_1 acetate. One vitamin A unit is to one provitamin A unit as 1 is to 2.[5, 6]

The standard β-carotene shall have a melting point of 180° (corr.) and show a light absorption at 465 mμ (in benzene) with an extinction of $\epsilon = 122{,}700$ ($E^{1\%}_{1\,cm.} = 2290$), or at 455 mμ (in cyclohexane) with $\epsilon = 130{,}800$ ($E^{1\%}_{1\,cm.} = 2400$). A solution of β-carotene in a vegetable oil serves as standard solution. Thus, according to U.S.P., 5 mg. of such a solution (β-carotene in cottonseed oil) contain 0.6 γ of β-carotene equal to 1 I.U.; this solution is stabilized with hydroquinone. The standard vitamin A acetate shall have a melting point of 57.8 to 59° and a light absorption at 325 mμ with an extinction of $\epsilon = 50{,}000$ ($E^{1\%}_{1\,cm.} = 1525$) in isopropanol. Vitamin A_1 alcohol shows an $E^{1\%}_{1\,cm.} = 1750$ at 328 mμ (depending on the solvent, the values for $E^{1\%}_{1\,cm.}$ can vary slightly). A solution of vitamin A acetate in a vegetable oil is used as a standard solution. The stabilization is accomplished by adding at least 0.1 % of tocopherol.[3] The standard solution of U.S.P. contains 0.344 γ of vitamin A acetate in 0.1 mg. of solution (cottonseed oil). The final fixation of the conversion factor (C.F.)—that is, the ratio of biological activity to the value $E^{1\%}_{1\,cm.}$ (328 mμ)—was arranged at the same conference.[3] Since 0.344 γ of vitamin A acetate equals 1 I.U., it can be stoichiometrically calculated that pure vitamin A alcohol contains 3.33×10^6 I.U. per gram. Based on the value of 1750 for $E^{1\%}_{1\,cm.}$ (328 mμ) for vitamin A_1 alcohol, a conversion factor of 1900 was ascertained. The conference declared this value to be obligatory. Before this final regulation the conversion factor had been subject to great fluctuations. Because of the existence of the so-called irrelevant absorption in fish liver oils, the value for $E^{1\%}_{1\,cm.}$ (328 mμ) was not clearly defined. In 1934 it was internationally set at 1600,[1] but in the United States the value of 2000 was in general use. Consequently 1 U.S.P. unit equaled 0.8 I.U.[7] With the international conversion factor fixed at 1900, which is generally accepted, such errors are removed. But it must be emphasized here that the conversion factor of 1900 can be correctly used only if the absorption curve obtained from the spectroscopic analysis is identical with that of the standard preparation (see also p. 90).

VI. Occurrence

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The provitamins A occur in the vegetable and animal kingdoms in greater quantities than vitamin A itself. Chemically they belong exclusively to the

[5] R. A. Morton, *J. Pharm. and Pharmacol.* **2**, 133 (1950).
[6] C. J. Koehn, *Arch. Biochem.* **17**, 337 (1948).
[7] See, e.g., E. M. Hume, *Nature* **151**, 535 (1943).

class of the carotenoids. Naturally occurring provitamins A are α-, β-, and γ-carotenes (here referred to mostly as carotene), cryptoxanthin, myxoxanthin, echinenon, aphanin, aphanicin, and torularhodin. It is not yet proved whether leprotin has the biological potency of vitamin A.[1]

Myxoxanthin is found in algae,[2] echinenon in the red fungus *Hymeniacidon sanguina*,[3] aphanin and aphanicin also in certain algae,[4] torularhodin in red yeasts,[5] and leprotin in *Mycobacterium phlei*[6] (for further reference, see Karrer and Jucker[7]). Beside these naturally occurring carotenoids there are known a great number of synthetical compounds and of degradation products of carotenoids, which are likewise biologically active.[8, 9] Regarding the formulas of vitamin A, of the natural provitamins A, and of the artificial products which are physiologically active, one should recognize that their potency depends on the existence of a β-cyclocitral ring system and of a side chain consisting of two isoprene residues. Only recently it has been found that the vitamin A_2 containing a dehydrocyclocitral ring also shows vitamin A activity.[10]

In contrast to the provitamins A, vitamin A is found exclusively in the animal organism; at least until now there has been no proof of the existence of vitamin A in vegetable material, either free or esterified. We may therefore assume that vitamin A is a secondary product and that the animal organism only is able to convert the provitamins A into vitamin A. It seems to be certain that fish are able to convert not only provitamins A but also other carotenoids (e.g., astaxanthin) into vitamin A.[11] Fish liver oils represent the richest and most important source for this vitamin.

β-Carotene is most widely distributed in nature, and since it invariably accompanies chlorophyll it is found in all green parts of plants. It occurs in abundance in carrot roots, paprika skin, sorb apples, oranges, spinach, sweet potatoes, and pumpkins. Furthermore it is found in many of the lower plants such as algae, in mushrooms, and also in bacteria[7] (see Table XVII).

Cryptoxanthin[7] is found preponderantly in Indian corn. Herbivorous animals receive their provitamin A exclusively from green plants. As do-

[1] Y. Takeda and T. Ohta, *Hoppe-Seyler's Z. physiol. Chem.* **267**, 171 (1941).
[2] J. M. Heilbron and B. Lythgoe, *Nature* **136**, 989 (1935); *J. Chem. Soc.* **1936**, 1376.
[3] P. J. Drumm, W. F. O'Connor, and L. P. Renouf, *Biochem. J.* **39**, 208 (1945).
[4] H. Tischer, *Hoppe-Seyler's Z. physiol. Chem.* **251**, 109 (1936); **260**, 257 (1939); **281**, 143 (1944).
[5] P. Karrer and J. Rutschmann, *Helv. Chim. Acta* **26**, 2109 (1943); **28**, 795 (1945); **29**, 355 (1946).
[6] Ch. Grundmann and Y. Takeda, *Naturwissenschaften* **25**, 27 (1937).
[7] P. Karrer and E. Jucker, Carotinoide. Verlag Birkhäuser, Basel, 1948.
[8] F. Bohlmann, *Z. angew. Chem.* **62**, 4 (1950).
[9] H. Vogel and H. Knobloch, Chemie und Technik der Vitamine. 3rd ed. p. 41, Ferdinand Enke, Stuttgart, 1950.
[10] E. M. Shantz, *Science* **108**, 417 (1948).
[11] J. A. Lovern, *Chemistry & Industry* **61**, 222 (1942).

TABLE XVII[9]

OCCURRENCE OF PROVITAMINS A IN FOOD

Vegetable	I.U./100 g.
Tomato squash	1,000
Chervil	10,000
Fresh leek leaves	6,650
Cabbage turnip leaves	10,000
Cauliflower leaves	13,000
Cauliflower	0
Rapeseed oil	650
Maize oil	400
Olive oil	160
Palm oil, yellow	1,000
Palm oil, red	2,000
See also carotene content of certain vegetables.[15-21]	

OCCURRENCE OF VITAMIN A[9]

Liver oils of	I.U./g.
Fresh-water perch	8,000
Chicken (normally fed)	20,000
Goose (normally fed)	14,000
Horned toad (*Lacerta ocellata*)	56,000
Basilisk (*Basiliscus americanus*)	120,000
Stereolepis gigas	600,000
Halibut	100,000
Codfish	600
Sea eel	4,200
Salmon	5,000
Turbot	5,000
Mackerel	130,000
Tunny	120,000
Swordfish	200,000

VITAMIN A CONTENT OF VARIOUS HUMAN ORGANS[9]

Organ	I.U./g. (moist substance)
Heart	1.4
Kidney	2.7
Brain	0.5
Liver	156
Lung	1.2
Stomach	1.3
Skin	1.2
Spleen	1.0
Skeletal muscle	0.5
Kidney capsule	6.0
Serum	5–10

mestic animals are largely fed with fodder, the drying process has been examined intensively with regard to the provitamin content.[12-14] In the United States, alfalfa, fresh and dry, plays a great role in supplying these animals with vitamins A.

In all plants the provitamins A are accompanied by carotenoids which are biologically inactive; this is of great importance for the chemical and physical analysis. Human and animal organisms show an accumulation of vitamin A in the liver and the kidneys as well as in the milk and in the blood (see Table XVII). Both vitamin A and carotene are found in the human and animal organism and its secretions along with biologically inactive carotenoids.[22]

Fish liver oils, being particularly rich in vitamin A, are the only natural source for commercial extraction. According to recent results, the livers of polar bears are the richest source of vitamin A known so far. They are so rich in vitamin A as to cause poisoning by hypervitaminosis; 0.5 to 0.7 g. of polar bear liver per rat may kill.[23] The reasons why these animals and fish store vitamin A in such large amounts are not yet known.

It is unlikely that the organism of the fish is able to carry out a true biosynthesis of vitamin A. It is assumed that fish ingest carotenoids with the food plankton and lower plants, algae, and crustaceans, and then convert these into vitamin A. The human being and the higher animals do not have an ability to transform carotenoids of non-provitamin A type into vitamin A. It seems to be proved that many carnivorous animals, e.g., cats, are not capable of transforming provitamins A into vitamin A.[24] Such animals have no other source for this vitamin than the organs and the blood of their prey. Milk is relatively rich in vitamin A and so are all fat-containing milk products. In this case the vitamin A content is subject to seasonal fluctuations depending on whether fresh (summer) or dry (winter) fodder has been

[12] A. R. Kemmerer and G. S. Fraps, *Ind. Eng. Chem. Anal. Ed.* **38**, 457 (1946).
[13] H. L. Mitchell and H. H. King, *J. Biol. Chem.* **166**, 477 (1946).
[14] H. L. Mitchell, W. G. Schrenk, and H. H. King, *Arch. Biochem.* **16**, 343 (1948).
[15] L. A. Moore, *Ind. Eng. Chem. Anal. Ed.* **12**, 726 (1940).
[16] H. C. H. Graves, *Chemistry & Industry* **61**, 8 (1942).
[17] F. P. Zscheile, B. W. Beadle, and H. T. Kraybill, *Food Research* **8**, 299 (1943).
[18] A. R. Kemmerer and G. S. Fraps, *Food Research* **10**, 457 (1945).
[19] A. R. Kemmerer, G. S. Fraps, and W. W. Meinke, *Food Research* **10**, 66 (1945).
[20] B. D. Ezell and M. S. Wilcox, *Food Research* **13**, 203 (1948).
[21] A. R. Kemmerer and G. S. Fraps, *Ind. Eng. Chem. Anal. Ed.* **15**, 714 (1943).
[22] H. von Euler, B. von Euler, and H. Hellström, *Biochem. Z.* **202**, 370 (1930); A. E. Gillam and M. S. El Ridi, *Biochem. J.* **29**, 2465 (1935).
[23] K. Rodahl and T. Moore, *Biochem. J.* **37**, 166 (1943); K. Rodahl, 18th International Physiological Congress, Copenhagen, 1950; *Z. angew. Chem.* **63**, 78 (1951).
[24] B. Ahmad, *Biochem. J.* **25**, 1195 (1931).

given.[25-27] The same applies to the vitamin A content in the yolk of eggs. Harms[28] made exhaustive researches on the amount of vitamin A in hens' eggs after different ways of feeding. In the vegetable cell the carotene is combined with proteins and lipoids.[29, 30] Also in blood serum carotene and vitamin A are combined with proteins.[31-33] In natural compounds vitamin A is very often found as the ester, e.g., in fish liver oils, among others, as palmitic acid ester.[34-36] In halibut liver oil up to 95 % occurs as ester,[37] but in egg yolk, 71 to 93 % is not esterified.[38] The esters of vitamin A are far more resistant to oxidative destruction than the free alcohol[39] and are therefore better for the vitaminization of foodstuffs.[40]

In blood about one-fifth of the total vitamin A content is present as ester. This figure rises with large vitamin A supplies, while the vitamin A alcohol level in the blood remains unchanged.[41]

The organs of fresh-water fish and also of sea fish that spawn in fresh water, such as salmon, contain vitamin A_2 which differs in its constitution from vitamin A by possessing one more double bond in the β-cyclocitral ring. This compound was formerly thought to have only minor biological potency.[42] Latest experimental results, however, indicate that vitamin A_2 is 40 % as active as vitamin A_1.[43] β-Carotene is converted within the organism of fresh-water fish into vitamin A_2.[44] The same applies most probably to other provitamins and carotenoids, whereas *in vivo* no conversion

[25] N. K. De, S. Ranganathan, and A. R. Sundararajan, *Indian J. Med. Research* **34**, 3 (1946).
[26] C. R. Barnicoat, *J. Dairy Research* **15**, 80 (1947).
[27] B. C. R. Sarkar, *J. Dairy Sci.* **31**, 165 (1948).
[28] F. Harms, *Vitamine u. Hormone* **2**, 36 (1942).
[29] R. Kuhn and H. J. Bielig, *Ber.* **73**, 1080 (1940).
[30] W. Straus, *Helv. Chim. Acta* **25**, 179, 489, 705 (1942); **26**, 1370 (1943).
[31] L. S. Palmer, *J. Biol. Chem.* **23**, 261 (1915).
[32] W. Kraus, Dissertation, University of Zürich, 1939.
[33] L. M. Działoszynski, E. M. Mystkowski, and C. P. Stewart, *Biochem. J.* **39**, 63 (1945).
[34] L. Reti, *Compt. rend. soc. biol.* **120**, 577 (1935).
[35] S. Hermano, *Sci. Papers Inst. Phys. Chem. Research (Tokyo)* **32**, 44 (1937).
[36] H. M. Kascher and J. G. Baxter, *Ind. Eng. Chem. Anal. Ed.* **17**, 499 (1945).
[37] K. Ritsert, *Vitamine u. Hormone* **3**, 57 (1943).
[38] A. W. Neff, D. B. Parrish, J. S. Hughes, and L. F. Payne, *Arch. Biochem.* **21**, 315 (1949).
[39] U. P. Basu and S. K. Sen-Gupta, *J. Am. Chem. Soc.* **70**, 413 (1948).
[40] J. G. Baxter and C. D. Robeson, *J. Am. Chem. Soc.* **64**, 2411 (1942).
[41] H. Popper, F. Steigmann, A. Dubin, H. Dyriewicz, and F. P. Hesser, *Proc. Soc. Exptl. Biol. Med.* **68**, 676 (1948).
[42] J. L. Jensen, E. M. Shantz, N. D. Embree, J. D. Cawley, and P. L. Harris, *J. Biol. Chem.* **149**, 473 (1943).
[43] E. M. Shantz, *Science* **108**, 417 (1948).
[44] R. A. Morton and R. H. Creed, *Biochem. J.* **33**, 318 (1939).

of vitamin A_2 into A_1 has been observed up to now.[45] Vitamin A is always accompanied by neovitamin A (13,14-mono-*cis* vitamin A). According to Harris and associates neovitamin A shows in the storage test (see p. 98) a vitamin A potency of 75% and in the growth test a potency of 85%.[46] The provitamins A too can be accompanied by stereoisomers, as was proved by Beadle and Zscheile.[47] Our knowledge of the carotenoid stereoisomers relates principally to the comprehensive and classical investigations of Zechmeister *et al.*[48-52] Deuel[53, 54] has stated that the stereomeric carotenes are not fully potent biologically. Work on this subject is still going on, and further results must be awaited before a definite knowledge in regard to this very complex and important problem is reached.

In whale liver oil a substance is found in considerable quantities which shows no physiological activity but which is changed into vitamin A by heating to over 170°. This substance in the crystalline state is called kitol.[55-58] An analogous substance has been isolated from fresh-water fish oils. Through heating it is changed into vitamin A_2 and is termed kitol$_2$.[59] Nothing definite is known about the role which kitol plays in the organism.

Vitamin A and its provitamins are chemically rather sensitive compounds which suffer irreversible changes under the influence of light and air.[60] Thus special attention should be paid to the conservation of foodstuffs and fodder containing these substances. Peroxides or peroxide-containing oils instantaneously destroy vitamin A;[61] also metal ions, especially copper and

[45] E. M. Shantz and J. H. Brinkmann, *J. Biol. Chem.* **183**, 467 (1950).
[46] P. L. Harris, S. R. Ames, and J. H. Brinkmann, *J. Am. Chem. Soc.* **73**, 1252 (1951).
[47] B. W. Beadle and F. P. Zscheile, *J. Biol. Chem.* **144**, 21 (1942).
[48] A. E. Gillam and M. S. El Ridi, *Nature* **136**, 914 (1935); *Biochem. J.* **30**, 1795 (1936); **31**, 251 (1937).
[49] A. Polgár and L. Zechmeister, *J. Am. Chem. Soc.* **64**, 1856 (1942); **65**, 1528 (1943); **67**, 108 (1945).
[50] L. Zechmeister and A. Polgár, *J. Am. Chem. Soc.* **66**, 137 (1944).
[51] L. Zechmeister, *Chem. Revs.* **34**, 267 (1944).
[52] F. P. Zscheile, *Arch. Biochem.* **5**, 77, 211 (1944).
[53] H. J. Deuel, Jr., C. Johnston, E. Sumner, A. Polgár, and L. Zechmeister, *Arch. Biochem.* **5**, 107 (1944); **6**, 157 (1945); **7**, 247 (1945).
[54] H. J. Deuel, Jr., C. Hendrick, E. Straub, A. Sandoval, J. H. Pinckard, and L. Zechmeister, *Arch. Biochem.* **14**, 97 (1947).
[55] N. D. Embree and E. M. Shantz, *J. Am. Chem. Soc.* **65**, 910 (1943).
[56] H. Pritchard, H. Wilkinson, J. R. Edisbury, and R. A. Morton, *Biochem. J.* **31**, 258 (1937).
[57] H. Willstaedt and H. B. Jensen, *Nature* **143**, 474 (1939).
[58] F. B. Clough, H. M. Kascher, C. D. Robeson, and J. G. Baxter, *Science* **105**, 436 (1947).
[59] N. D. Embree and E. M. Shantz, *J. Am. Chem. Soc.* **65**, 910 (1943).
[60] R. A. Bolomey, *J. Biol. Chem.* **169**, 323, 331 (1947).
[61] G. Carrara and A. Mandella, *Vitaminologia* 33 (1944).

cobalt, accelerate the oxidative destruction in fish liver oils.[62] Ultraviolet irradiation[63] produces the same results. Extensive researches on the enzymatic oxidation of carotene, for which the presence of unsaturated fatty acids is necessary, have been published.[64-66]

Many fatty oils originating from plants contain natural antioxidants, but in most cases they are added during the process of preservation. The tocopherols,[67] palmitoyl ascorbic acid, hydroquinone,[68] gossypol, and others[69-71] are used for this purpose. In order to preserve the biological potency of foods they must be stored at the lowest temperature possible. On the other hand, it is necessary to destroy enzymes by heating (autoclaving) for a short time before drying foods.[72] The production of dried eggs must also be done with the greatest care. About 30 to 50% of the vitamin A is destroyed if the egg yolk is dried in the ordinary way; vacuum drying under carbon dioxide reduces the loss to 5 to 7%.[73]

VII. Effects of Vitamin A Deficiency and Hypervitaminosis A in Animals

S. BURT WOLBACH

A. GENERAL CONSIDERATIONS

The morphologic effects of vitamin A deficiency are found in many epithelial structures and in epiphyseal cartilage of growing bones. These effects in young animals are present before the rate of growth is materially retarded and may be regarded as specific or primary consequences of the deprivation. Administration of vitamin A in great excess (250 to 1000 I.U. per gram) in an early period of growth results in a remarkable acceleration of epiphyseal cartilage cell sequences and of the remodeling of bones which normally accompany skeletal growth.

[62] W. Heimann, *Deut. Lebensm. Rundschau* **43**, 35 (1947).
[63] T. A. Chalmers, T. W. Goodwin, and R. A. Morton, *Nature* **155**, 513 (1945).
[64] J. B. Sumner and A. L. Dounce, *Enzymologia* **7**, 130 (1939).
[65] H. Süllmann, *Helv. Chim. Acta* **24**, 646 (1941).
[66] E. L. Hove, *Science* **98**, 433 (1943).
[67] L. O. Buxton, *Ind. Eng. Chem.* **39**, 225, 1171 (1947).
[68] H. N. Holmes, R. E. Corbet, and E. R. Hartzler, *Ind. Eng. Chem.* **28**, 133 (1936).
[69] E. L. Hove, *J. Biol. Chem.* **156**, 633 (1944).
[70] P. B. Müller, *Mitt. Gebiete Lebensm. u. Hyg.* **39**, 275 (1948).
[71] H. L. Mitchell, W. G. Schrenk, and H. H. King, *Mitt. Gebiete Lebensm. u. Hyg.* **41**, 570 (1949).
[72] R. C. Mills and E. B. Hart, *J. Dairy Sci.* **28**, 339 (1945).
[73] G. Wendland, *Vitamine u. Hormone* **5**, 46 (1944).

The impressive changes in morphology and structure of tissues which remain viable, though profoundly altered in function, suggest that the role of vitamin A is somewhat in character of a hormone or of an inductor or evocator. Actually, the epithelial changes attending vitamin A deficiency and recovery therefrom duplicate accurately those of the vaginal epithelium accompanying the estrous cycle of mammals. Hypervitaminosis A bone effects, like the patterned growth of fetal bone, are unrelated to concurrent functioning though conforming to normal growth patterns. In general, the morphologic results of vitamin A deficiency are most pronounced upon growth and differentiation of tissues—in growing and adult animals by keratinization of epithelia which are renewed by multiplication of basal cells, in fetal life by many malformations, and in early postnatal life by retardation of skeletal growth throughout the period of growth. Skeletal growth may be retarded by deficiency of vitamin A beyond the normal growth period of a species and be resumed following vitamin A administration.

Of interest is the fact that all vertebrates require an outside source of vitamin A or a carotenoid precursor, for vision, for maintenance of many epithelia, and for tooth and skeletal growth. This holds true for all animals thus far tested—man, monkeys, pig, cattle, dog, fox, rabbit, guinea pig, rat, mouse, fowl.[1, 2] The hypervitaminosis A effects upon skeletal growth have been observed in man, dog, guinea pig, rat, mouse, and fowl.[3, 4]

B. VITAMIN A DEFICIENCY, POSTNATAL

1. Epithelial Tissues and Organs

Epithelia of ectodermal, mesodermal, and entodermal origin are similarly affected. The results summarized are: atrophy, reparative proliferation by basal cells, and growth and differentiation of the new cells into a stratified keratinizing epithelium. The replacement epithelium, regardless of the original function and structure of the region, is identical in all locations and comparable in all its layers with epidermis.[1, 2, 5] Disregarding semantics, for the sake of brevity I shall refer to the sequences which result in formation of the replacement epithelia as keratinizing metaplasia. The result of repair induced by vitamin A administration is that, regardless of the identical composition of the replacement epithelium, there is return to normal in morphology and function for each region.[6]

[1] S. B. Wolbach, *J. Am. Med. Assoc.* **108**, 7 (1937).
[2] R. H. Follis, Jr., The Pathology of Nutritional Disease. Charles C Thomas, Springfield, Ill., 1948.
[3] S. B. Wolbach, *J. Bone and Joint Surg.*, **29**, 171 (1947).
[4] C. L. Maddock, S. B. Wolbach, and S. J. Maddock, *J. Nutrition* **39**, 117 (1949).
[5] S. B. Wolbach and O. A. Bessey, *Physiol. Revs.* **22**, 233 (1942).
[6] S. B. Wolbach and P. R. Howe, *J. Exptl. Med.* **57**, 511 (1933).

The order of the deficiency responses of organs varies somewhat with the species of animal, including man, but the same organs are involved and are presented here roughly in order of sequence of appearance of keratinizing metaplasia: salivary glands, including the submaxillary, parotid, and all accessory glands of the buccal cavity, tongue, and pharynx; the respiratory tract, including the nares and accessory sinuses; Jacobson's organ, trachea, and bronchi; genitourinary tract, including the bladder, ureter, renal pelvis, epididymis, prostate, seminal vesicles, coagulating gland, uterus, oviduct, and accessory sex glands of the vulva; eyes and paraocular glands, including

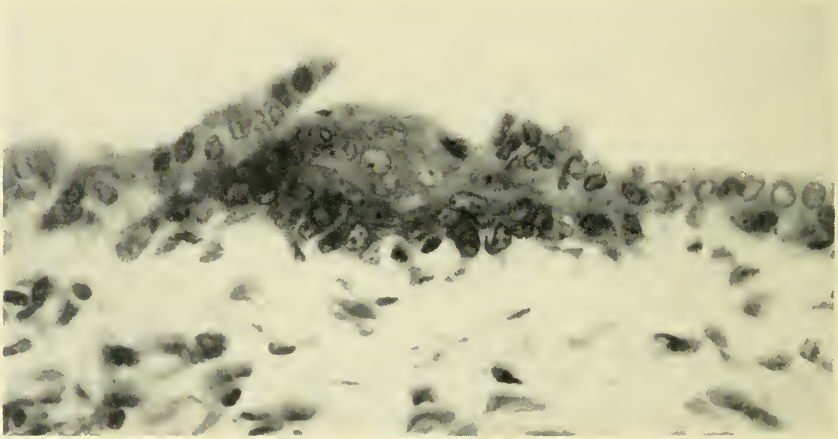

FIG. 26. Vitamin A deficiency in the rat. Duct of the submaxillary gland showing a focus of reparative proliferation with early undermining and keratinizing metaplasia.

the corneal and palpebral conjunctivae, the Harderian, intraorbital and extraorbital lacrymal glands, and the Meibomian glands; the skin.

In general, in vitamin A deficiency, the epithelia which atrophy and which become replaced by stratified keratinizing epithelium are those which have a secreting function in addition to the role of a covering layer and whose functioning cells are without power to divide. Replacement, therefore, takes place from focally distributed basal cells which multiply, spread beneath the original epithelium, and finally, through coalescence of areas thus produced and differentiation, form a continuous epidermis-like layer (Figs. 26, 27, and 28). Epithelial cells with more complex physiological roles and power to divide, such as liver, renal tubules, adrenals, and anterior hypophysis, do not exhibit early severe atrophy, nor are they replaced by keratinizing epithelium. The stratified and transitional epithelia—cornea, renal pelvis, ureter, and bladder—become hyperkeratotic, all of the continuous layer of basal cells participating in the process. Presumably these epithelia have

FIG. 27. Vitamin A deficiency in the guinea pig. Uterus. Total keratinizing metaplasia of the epithelium lining the cavity, and of the glands.

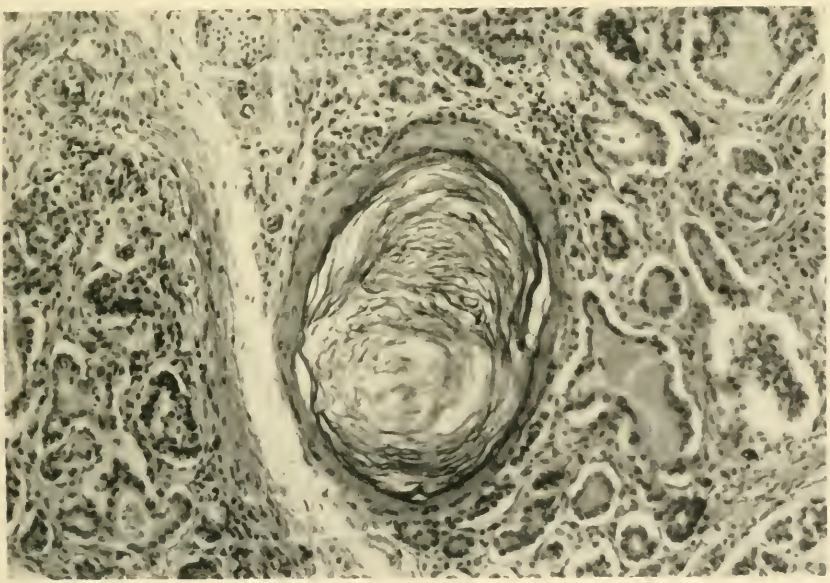

FIG. 28. Vitamin A deficiency in the human infant. A duct in the pancreas lined with stratified keratinizing epithelium.

specialized functions which are suppressed by the deficiency. No satisfactory explanation can be offered for the fact that the reparative activities of basal cells of many different epithelia in vitamin A deficiency end in an identical stratified keratinizing epithelial product comparable in all details with epidermis.

The gross pathologic features in man and animals are largely the result of the accumulation of keratinized epithelial cells in glands and their ducts, in other organs, and in conjunctival sacs. Cysts of considerable size, filled with yellowish cheesy masses of keratinized cells, may be formed in many glands. In the lungs of human infants and in experimental animals this process produces occlusion of bronchi, atelectasis, and formation of bronchiectatic cavities filled with keratinized cells. Early workers regarded such cysts and bronciectatic cavities as abscesses, and therefore vitamin A was regarded as an anti-infective vitamin. The plugs of desquamated epithelial cells in ducts, trachea, and bronchi which communicate with regions normally harboring bacteria act as a culture medium, yet rarely in man and experimental animals does invasion of tissue result, presumably because of the barrier presented by the stratified epithelium.

Because most features of the gross and microscopic pathology of vitamin A deficiency are the result of keratinizing metaplasia, detailed accounts of the various tissues and organs of the body will not be presented. Emaciation—a consequence of most severe deficiencies—may be taken for granted. The effect upon skeletal growth and consequent damage to a growing central nervous system will be presented in some detail because of confusion, now current in the literature, of the mechanisms involved and of cause and effect.

a. Eye

The night blindness of vitamin A deficiency in the adult is a reversible phenomenon and is probably unaccompanied by morphologic changes demonstrable by available techniques. Irreversible loss of vision does occur in young animals and is fully explained by pressure effects upon optic nerve and blood vessels, resulting from retardation of the growth of the skull. Johnson[7] has carefully studied the retina of rats placed upon a vitamin A-deficient diet at 23 days of age. The earliest change was edema of the retina; then followed slight degenerative changes of the outer segments of rods. Recovery after replacement therapy was rapid. A later lesion, complete degeneration of the outer segments of rods "(and possibly the inner sigments as well)," required a longer period of therapy. Lesions involving "the greater part of the outer nuclear layer, or more" proved to be irrep-

[7] Myra L. Johnson, *Arch. Ophthalmol. (Chicago)* **29**, 793 (1943).

arable. The sequences described by Johnson coincide so well with the gradual increase of intracranial pressure resulting from retardation of growth of the skull and unretarded growth of the nervous system that a mechanical explanation seems unavoidable.[8]

The epithelia of cornea and bulbar and palpebral conjunctivae become keratinized. Blood vessels grow into the tunica propria of the cornea from the limbic plexus concurrently with the epithelial changes, and are found throughout the thickness of the cornea. Keratinizing metaplasia of the ducts and atrophy of the lacrimal glands, together with the conjunctival changes, result in loss of all secretions—xerosis. Accumulations of keratinized cells in the conjunctival sac excite inflammatory reactions—xerophthalmia. Edema and necrosis of the cornea—keratomalacia—may occur beneath an intact hyperkeratotic epithelium. Bacterial infection favored by the accumulation of keratinized cells results in ulceration of the cornea and consequently hypopyon. On the whole, the gross ocular manifestations in animals and in man appear late in the deficiency.[9, 10]

b. Respiratory Tract

Keratinizing metaplasia of the entire respiratory tract, nares to bronchioles, occurs early. Cough is an early symptom in infants. Atrophy of the epithelium and loss of ciliary action even before replacement by keratinizing epithelium favor bacterial invasion, and hence lobular pneumonia has been a frequent immediate cause of death.[9] In deficient rats foreign particles—starch granules from the food, for example—are frequently found in the alveoli of lungs. The development of bronchiectases, atelectasis, and keratinizing walled cysts are results inherent in the specific epithelia response of the deficiency, as are involvement of the Eustachian tubes, middle ears, and accessory respiratory sinuses.

c. Alimentary Tract

The epithelia of the mucosa of stomach and small and large intestines do not undergo keratinizing replacement; at most, slight degrees of atrophy of gastric and intestinal glands can be found. The esophagus becomes hyperkeratotic. Keratinizing metaplasia of pancreatic ducts occurs but not of biliary ducts (see Fig. 28). The enamel organ of forming teeth in the human, and throughout life of the incisor teeth of rats and guinea pigs and presumably of all rodents, undergoes atrophy and keratinizing metaplasia; the consequences will be described in succeeding pages.

[8] S. B. Wolbach and O. A. Bessey, *Arch. Pathol.* **32**, 689 (1941).
[9] K. D. Blackfan and S. B. Wolbach, *J. Pediat.* **3**, 679 (1933).
[10] L. K. Sweet and H. J. K'ang, *Am. J. Diseases Children* **50**, 699 (1935).

d. Genitourinary Tract

Keratinizing metaplasia in all species occurs in the renal pelvis, ureters, bladder, epididymis, uterus (see Fig. 27), oviduct, and accessory sex glands in all species. Obstruction of the urinary system, the result of the accumulation of keratinized cells, may be the immediate cause of death in rats, and grossly visible lesions of this character have been found in human infants.[9, 10] Other demonstrated sites of keratinizing metaplasia in the human are uterus, epididymis, Fallopian tube, bladder, and vagina. In the urinary tract desquamated keratinized cells may become the nidus of calculus formation, but otherwise there is no direct relation of vitamin A deficiency to calculus formation. Atrophy of the seminiferous tubules is also a result of vitamin A deficiency in man and laboratory animals, but keratinizing metaplasia does not occur. The undifferentiated sex cells—spermatogonia—remain viable so that the atrophy, unlike that in vitamin E deficiency, is reversible.[6, 11, 12]

No effect of vitamin A upon the ovaries and/or ova has been described. None has been found in human infants or in laboratory animals by the writer.

e. Hematopoietic System

No specific features have been described in man or laboratory animals in relation to the anemia, the atrophy of blood-forming bone marrow, and the atrophy of lymphoid tissues—spleen, lymph nodes, and thymus. There is, however, a greater deposit of hemosiderin in liver and spleen than that which accompanies other vitamin deficiencies. The Hassall corpuscles of the thymus become enlarged and distended with keratinized epithelial cells. In animals, following the outburst of hematopoiesis in bone marrow and spleen produced by resumption of vitamin A feeding, the hemosiderin rapidly disappears.

f. Skin

The conspicuous hyperkeratosis of hair follicles reported chiefly from China[10, 13] but also from other countries[14] rarely appears before puberty in vitamin A-deficient humans. With it is associated atrophy and hyperkeratinization of sebaceous glands and ducts of sweat glands. Although large amounts of vitamin A unquestionably cause restoration to normal, the precise relation of the hair follicle hyperkeratosis to vitamin A is confused.

Hyperkeratotic papules of hair follicle origin occur in scurvy of adults[13]

[11] K. E. Mason, *J. Exptl. Zool.* **55**, 101 (1930).

[12] M. M. Sampson and V. Korenchevsky, *J. Pathol. Bacteriol.* **35**, 875 (1932).

[13] C. N. Frazier and C. K. Hu, *Arch. Dermatol. and Syphilol.* **33**, 825 (1936).

[14] O. A. Bessey and S. B. Wolbach, The Vitamins, Chapter 11, p. 27. American Medical Association, Chicago, Ill., 1939.

and were described in a case of experimental human scurvy in the presence of an adequate vitamin A intake.[15] Microscopic hyperkeratosis of hair follicles and epidermis and absence of sebaceous glands and edema and homogenization of the elastic tissue and of collagen fibers are described by H. Montgomery in a single biopsy specimen from a physician who voluntarily remained on a vitamin A-free diet for 189 days. Another biopsy taken 17 days after vitamin A therapy was instituted showed normal skin.[16] In animals, the skin responses to vitamin A deficiency have been reported as atrophy of the epidermis and its appendages. No connective tissue changes have been described. A review of the writer's extensive material from guinea pigs and rats fails to show significant degrees of hyperkeratosis, and certainly no plugging of hair follicles. On the other hand, there is atrophy of the hair bulb and of internal sheath cells in newly formed hair follicles. Some confusion has arisen, in regard to descriptions of both human skin and animal skins because of the authors' lack of knowledge of the hair follicle cycle in relation to hair formation.[17] Sullivan and Evans succeeded in producing in rats some degree of hyperkeratinization of epidermis and hair follicles by withdrawing vitamin A from rats previously subjected to a diet deficient in the heat-stable members of the vitamin B group.[18]

Although the morphologic changes of the epidermis are not impressive, this tissue should be of major importance in endeavors to elucidate the action of vitamin A because it has precisely the structure which so many epithelia adopt as a specific consequence of the deficiency.

g. Repair of Epithelia in Recovery from Vitamin A Deficiency

Repair in rats after they are restored to a normal diet is rapid and may be visible in 5 days. More rapid results might be obtained now that massive doses in the form of concentrates or crystalline vitamin A are available. Repair is presumably initiated by the lowermost layer of cells of the replacement epithelium, all of which have proliferative powers as in the stratum germinativum of epidermis. This layer, one to two cells deep, remains intact; immediately above it, cells, presumably irreversibly headed toward keratinization, become vacuolated and invaded by polymorphonuclear leucocytes, suggesting that both autolysis and heterolysis account for the disappearance of the middle stratum of cells. The upper layer of keratinized cells disappears completely; the lowermost layer proliferates and differentiates into epithelium normal for the region. The sequences duplicate accurately those of the rat's vagina in diestrus.

[15] J. H. Crandon, C. C. Lund, and D. B. Dill, *New Eng. J. Med.* **223**, 353 (1940).
[16] L. F. Steffens, H. I. Bair, and C. Sheard, *Am. J. Ophthalmol.* **23**, 1325 (1940).
[17] S. B. Wolbach, *Ann. N. Y. Acad. Sci.* **53**, 517 (1951).
[18] M. Sullivan and V. J. Evans, *Arch. Dermatol. and Syphilol.* **51**, 17 (1945).

As the epithelial changes in vitamin A deficiency and in recovery form a cycle that could not happen in animals in natural habitat, it is of interest to note that the recovery phenomena cannot be those of a pattern established phylogenetically, but they do follow a familiar mammalian physiological pattern.

h. Teeth

Vitamin A deficiency produces severe defects in the incisor teeth of rats and guinea pigs because, as in all rodents, these teeth are continuously growing at a rapid rate, and throughout life the odontogenic epithelium persists and remains active both as tooth organizer and producer of enamel. On the labial side the odontogenic epithelium extends for the entire length of the incisor tooth as the enamel-forming organ; on the lingual side it extends only for a short distance, acts only as an organizer, and forms no enamel. The primary consequence of vitamin A deficiency is upon the odontogenic epithelium. At the basal formative end of the tooth, growth continues but differentiation is incomplete and there is loss of organizing influence which results in inadequate and defective dentine formation associated with atrophic odontoblasts. The continuation of the growth of the odontogenic epithelium, because of the excessively thin dentine, may cause columns of epithelial cells to press into the pulp. Another consequence of the defective formation of dentine is plication and buckling of the wall of dentine near the formative end. More distally, the rate of dentine formation on the lingual side and lateral sides of the tooth is greatly retarded, and the odontoblasts are without orderly arrangement and resemble osteoblasts. On the labial side, where there has been enamel deposited, the odontoblasts remain columnar in shape and ample dentine continues to be deposited until atrophy of the enamel organ is complete. The vitamin A-deficient rodent incisor tooth has, therefore, a distinctive structure characterized by relatively great thickness of dentine on the labial side and excessively thin dentine elsewhere (Fig. 29). Complete organ atrophy results in loss of odontoblast morphology on the labial side also. Wolbach and Howe[19] called attention to the continuous organizing influence of the enamel-forming epithelium through the growth of the incisor tooth, and they characterized the odontoblast as a polarized osteoblast which lost its polarity following atrophy of the odontogenic epithelium. Depolarization of the odontoblasts and distortions of the tooth as a whole at its base or formative end are other consequences, all of which are specific results of suppression of the odontogenic epithelium functions of tooth organizer and enamel producer. These generalizations are also those of Pohto[20] and Schour

[19] S. B. Wolbach and P. R. Howe, *Am. J. Pathol.* **9**, 275 (1933).

[20] M. Pohto, Dissertation Med. Chem. Lab. und Odont. Inst., University of Helsinki, 1938.

FIG. 29. Vitamin A deficiency in the rat. Upper incisor tooth in early repair following restoration of vitamin A to the diet. Note the persistence of columnar odontoblasts and thickness of dentine in relation to the odontogenic epithelium on the labial side. Elsewhere the dentine is thin or even absent, with plication and inclusions of odontogenic epithelium. The odontoblasts, in spite of some "predentine" formation, have not yet resumed normal appearance.

et al.[21] Repair sequences are simple and dramatic. Restoration of function and morphology of the odontogenic epithelium is followed by the resumption of morphology and dentine deposition by odontoblasts. The repair

[21] I. Schour, M. M. Hoffman, and M. C. Smith, *Am. J. Pathol.* **17**, 529 (1941).

sequences serve to identify inclusions of odontogenic epithelium in the pulp by restored morphology of the cells and calcareous deposits. They also explain, as the result of bending and folding of the odontogenic epithelium at or near the formative end, through resumption of normal processes, the tooth duplications and tumor-like formations reported by Burn *et al.*[22]

Changes in a tooth "germ" of a human infant who succumbed to vitamin A deficiency, comparable in nature to those of rodent incisor teeth, have been described.[23]

Thus far, no relation between endocrine physiology and the specific morphologic manifestations of vitamin A deficiency has been established. Ovaries and testes have been mentioned above.

i. Endocrine Organs

The adrenals, the islands of Langerhans, and the thyroid and parathyroid glands undergo no noteworthy morphologic change. In spite of the severe inanition of long-continued vitamin A deficiency, the adrenals remain small and show, in both rats and guinea pigs, some evidences of atrophy in contrast to the enlargement resulting from simple underfeeding, particularly in guinea pigs.[24, 25]

The adrenal and thyroid glands will be considered briefly in the section on hypervitaminosis A.

2. Bone, Postnatal

After growth of the skeleton is completed, vitamin A deficiency has no specific effect upon bone structure. In growing animals—rats, guinea pigs, mice, dogs, and fowl—there are prompt and specific effects which result in retardation of skeletal growth and interference with growth patterns of bone which are distinguishable from the consequences of inanition, whether produced by inadequate amounts of a complete diet or by other vitamin deficiencies. Deficiency of vitamin A is the only known means of causing severe retardation of skeletal growth before causing appreciable retardation of growth of soft tissues, including the nervous system.[3, 8]

Skeletal growth changes in the human infant due to vitamin A deficiency have not been studied. The following account is based upon extended studies on albino rats and has been confirmed for the guinea pig, the dog, the duck, and the chick. Epiphyseal cartilage sequences are promptly retarded and cease entirely in long-continued A deficiency. All growth of the skeleton dependent upon replacement or endochondral bone formation

[22] C. G. Burn, A. U. Orton, and A. H. Smith, *Yale J. Biol. and Med.* **13,** 817 (1941).
[23] P. E. Boyle, *J. Dental Research* **13,** 39 (1933).
[24] H. T. Blumenthal and L. Loeb, *Am. J. Pathol.* **18,** 615 (1942).
[25] R. Whitehead, *J. Pathol. and Bacteriol.* **54,** 169 (1942).

ceases. Cells of the epiphyseal cartilage stop dividing, and cells which have almost reached full size undergo no further growth and may atrophy. Cells which have progressed to the vesicular stage complete their cycle and disappear, permitting penetration of blood vessels and osteoblasts from the medulla. Cartilage previously calcified is removed by osteoclasis. The matrix of the atrophic epiphyseal cartilage becomes calcified for a depth of

FIG. 30. Tibia-fibula complex of three rats of the same age. On the left, that of a riboflavin-deficient rat; on the right, that of a normal rat; in the center, that from a vitamin A-deficient rat. Note the convexity and thickness of the upper end of the tibia, the result of failure of remodeling sequences. Compare also the fibulae.

a few cells on the diaphyseal border, and in long-continued experiments a thin plate of bone is formed across the face of the epiphyseal disc. The result is similar, if not identical, to that caused by inanition from underfeeding and by deficiencies of other vitamins not specifically affecting bone growth, such as riboflavin and pyridoxine, extensively studied in the laboratory of the writer. Two facts distinguish the effects of vitamin A deficiency, one mentioned in a previous paragraph (the continued growth of soft tissues, including the nervous system), the other the cessation of remodeling sequences, while appositional bone formation continues. In the second category are included greatly diminished resorption of trabecular bone,

retardation and failure of Haversian system formation resulting in arrestment of compact bone formation, and continuation of appositional bone formation. The total effect is that of thicker, coarser, and shorter bones, less graceful in appearance than corresponding normal bones because of failure of resorption, wherever that is scheduled as a feature of normal growth, conspicuously at the ends of long bones (Fig. 30). In regions where appositional bone formation is the normal process by which bony contours are formed, we find apparently greater rate of bone production. In the long bones such regions are, for example in the rat, the third trochanter of the femur, the crest of the tibia, and the interosseous crests of tibia and fibula. In vitamin A deficiency, with cessation of longitudinal growth these regions present an appearance of excessive rate of growth. Serial sections of petrous bones of normal and vitamin A-deficient rats and dogs have shown that the activity or rate of appositional bone formation varies greatly in different regions and that the apparently excessive local bone growths are correlatable with the normal rates and patterns of growth. The petrous bone is an interesting, though difficult, region to study because the bony labyrinth of the internal ear is adult size at birth and because it does not undergo remodeling with increase in size of the base of the skull but does change position, moving outward and backward with reference to the body axis. Increase in size and remodeling of the temporal bone complex are features of its growth which are very evident in the recess for the paraflocculus in which appositional bone growth is much greater on the internal (medial) side than on the external (lateral) side. The reverse applies to bone resorption in the recess. The normal pattern of growth of the petrous bone of the rat is somewhat different from that of the dog; in the latter, in vitamin A deficiency, continuation of appositional bone growth causes compression of the eighth nerve, but not in the rat, although comparable building up of bone occurs in a closely adjacent situation. Thus the untoward consequences of changes in the skull vary with the species. Deafness in dogs[26, 27] and blindness in calves[28] are early results of the deficiency. Neither occurs in the rat.

In an impressive study of the ear in rabbits with long-continued vitamin A deficiency, Perlman[28a] has reported the formation of nodular bone formations in the internal auditory meatus, the cribiform plate, the cochlear aqueduct, and the modiolus within the cochlea which cannot be related to normal growth patterns but which can be related to lesions produced in guinea pigs by acidosis and ligation of blood supply reported by other

[26] E. Mellanby, *J. Physiol. (London)* **94**, 380 (1938).
[27] E. Mellanby, *J. Physiol. (London)* **99**, 467 (1941).
[28] L. A. Moore, *J. Nutrition* **17**, 443 (1939).
[28a] H. B. Perlman, *Arch. Otolaryngol.* **50**, 20 (1947).

workers. According to Perlman the sites of these bony nodules are determined by the formation of islands or buds of vascular connective tissue. A good list of references accompanies his article.

Mellanby,[29] who worked almost exclusively with dogs, attributes the skeletal changes resulting from vitamin A deficiency solely to its effect upon "the position and activity of osteoclasts and osteoblasts." Although in general he found great diminution in osteoclasis while osteoblastic activities remained essentially unchanged, thus accounting for the greater thickness of bones and alterations in contours, he made no attempt at correlation with endochondral bone growth or with normal growth patterns.

After vitamin A therapy, bone growth sequences return to normal. The order of resumption of the normal processes concerned has not been determined. Mellanby[29] has described only the early return of normal osteoblastic and osteoclastic activities of bone formed during the deficiency state. That prompt return to normal of epiphyseal cartilage sequences in the rat takes place has been shown by Wolbach and Bessey[8] by the fact that vitamin A therapy begun 7 to 10 days before the usual onset of paralyses prevents sufficient disparity of growth required for pressure effects of bone upon the nervous system. Rats prevented from paralyses in experiments of this type have nevertheless shown slight buckling of nerve roots within the spinal canal. The epiphyseal cartilage cells in all layers return to normal by resumption of mitotic activities, growth, and maturation. If, as occurs in long-continued vitamin A deficiency, a plate of bone has formed on the diaphyseal side, it becomes perforated and blood vessels penetrate the spaces occasioned by the degeneration of mature cartilage cells.[3]

3. Bone Growth and the Nervous System

No proof exists that degeneration of the nervous system results from vitamin A deficiency. The long-continued deficiency in fully grown animals does not result in disabilities of nervous origin or demonstrable lesions of the nervous system. In young animals, during the period of rapid growth, damage to the nervous system results from compression of brain, spinal cord, and nerve roots caused by the retarded skeletal growth already described.

The cranial cavity and spinal canal fail to enlarge sufficiently to accommodate the central nervous system, which continues to grow at a normal rate. The result in the cranial cavity is compression of the brain and its dislocation toward the foramen magnum and multiple herniations of the cerebrum and cerebellum into the venous sinuses of the dura at sites of

[29] E. Mellanby, A Story of Nutritional Research: The Effect of Some Dietary Factors on Bones and the Nervous System. Williams and Wilkins Company, Baltimore, 1950.

arachnoidal villi (Figs. 31, 32, and 33). The production of vitamin A deficiency is, in the opinion of the writer, an easy and accurate method of determining the sites of arachnoidal villi which vary in different species. In the spinal canal, deficiency of vitamin A results in overcrowding of its contents. Those dorsal and ventral nerve roots in the rat and guinea pig

Fig. 31. Vitamin A deficiency in the rat. Brain of a 9-week-old rat which had shown paralysis for 16 days. Herniations (H) of cerebrum and cerebellum into the transverse sinuses and confluens sinuum. Note flattening of the cerebellum where it was forced into the foramen magnum.

which arise distal (caudad) to the midthoracic region and leave the spinal canal at lower levels (lumbar and sacral) become too long for the intervertebral distances between levels of origin and exit. The result is buckling and herniation into intervertebral foramens and into posterior root ganglia (Fig. 34). Buckling of nerve roots on the ventral side of the spinal cord causes pressure atrophy of bone and herniations into the bodies of vertebrae (Fig. 35). In rat, guinea pig, and chick,[29a] appositional growth of bone

[29a] Since the completion of this manuscript, studies have been published on the effects of vitamin A deficiency and hypervitaminosis A on chicks and ducks. Re-

in the deficiency is not a cause of pressure upon spinal nerves at foramens of exit, as is the case in dogs.[27, 29] The histologic features of the herniations of brain and cerebellum studied in the rat[5] are those attending mechanical

FIG. 32. Vitamin A deficiency in the chick. Herniations from the olfactory lobes via arachnoidal villi into a confluens sinuum (undescribed) formed by the confluence of the saggital sinuses and veins from the orbits and surfaces of the cerebral hemispheres.

tardation of endochondral bone formation occurs promptly in these avian species with the result that there is compression of the spinal cord and brain. Herniations into arachnoidal villi of ducks and chicks are prominent features of the disproportionate growth of the central nervous system and the skull. Excessive vitamin A results in the same type of acceleration of endochondral bone growth as described for mammalian species.

Fig. 33. Vitamin A deficiency in the rat. Herniation of the cerebellum into the transverse sinus via an arachnoidal villus. Penetration of the calvarium at the site of an emissary vein.

Fig. 34. Vitamin A deficiency in the rat. Nerve root levels of sixth lumbar ganglion and first sacral ganglion. The buckled nerve roots have been dislodged from the intervertebral foramina and from a pitted vertebral body. From a 9-week-old rat which had shown paralysis for 7 days.

injury. They contain both cortical and medullary substance showing all degrees of degeneration, necrosis, and the usual cellular responses on the

FIG. 35. Vitamin A deficiency in the rat. Cross section of a lumbar vertebra showing pits occupied by buckled nerve roots. From a 9-week-old rat which had shown paralysis from 8 to 10 days.

part of neuroglia and phagocytic cells. A description of the nerve root herniations and associated peripheral nerves would include all the classic features of Wallerian degeneration. Evidence that the axons are not primarily impaired by the deficiency was shown by the fact that, proximal

to the injury of the nerve roots, the nerve fibers responded by regeneration and exhibited all the details described by Cajal in his classic studies of degeneration and regeneration produced experimentally by crushing injuries.

C. HYPERVITAMINOSIS A

1. Pathologic Physiology

Vitamin A, when given in excessive amounts, causes severe untoward results usually referred to as toxic. Instances of severe illness in adults, prompt onset of headache, vomiting, diarrhea, and giddiness, and, about a week later, desquamation of the skin and some loss of hair are on record as the result of ingesting 300 to 500 g. of polar bear liver, the vitamin A content of which may be as high as 18,000 I.U. per gram.[30]

Numerous instances (eighteen) of the deleterious effects of long-continued administration of vitamin A in the form of fish liver oil concentrates have been described in infants and young children, and but one instance in an adult who received a vitamin A preparation free from vitamin D. Symptoms and signs common to all these patients were scaly dermatitis, patchy loss of hair, fissured lips, irritability, anorexia, and skeletal pain. Nothing is known of the biologic properties of vitamin A that will explain the physiologic disturbances responsible for the "toxic" effects of hypervitaminosis A.

Laboratory studies of human patients to date have not given consistent results. With but one exception the source of vitamin A has been fish liver concentrates with a high vitamin D content, and therefore a new appraisal is necessary of the reported instances of lowered serum proteins, elevated alkaline phosphatase, and elevated serum lipids. On the whole, no definitive information has been obtained from laboratory animals given excessive amounts of vitamin A preparations free of vitamin D. There are no significant changes in calcium, phosphorus, and phosphatase values, which is not surprising because the skeletal effects to be described are those of increased tempo of growth processes in which resportion of bone is balanced by new bone formation. Cholesterol and phospholipid studies likewise have not been informative.[4]

Excessive doses of pure vitamin A in the rat increase the prothrombin time[31] and, although rats synthesize vitamin K in their intestinal tracts, will cause death from hemorrhage in about 90 % of rats on a vitamin K-free diet.[32] Discovery that excessive amounts of vitamin A cause prompt reparative changes in the rachitic metaphysis of the rat[33] led to metabolism studies

[30] K. Rodahl, *Norsk Polarinstitutt. Skrifter* **92**, (1949).
[31] S. E. Walker, E. Eylenburg, and T. Moore, *Biochem. J.* **41**, 575 (1947).
[32] C. L. Maddock, S. B. Wolbach, and D. Jensen, *Federation Proc.* **7**, 275 (1948).
[33] S. B. Wolbach and C. L. Maddock, *Federation Proc.* **8**, 376 (1949).

of rachitic rats before and after excessive vitamin A administration. The findings resulting from the vitamin A excess were a negative nitrogen balance, an excess of calcium excretion over intake, and a prompt marked negative phosphorus balance.[34]

A specific interrelation between vitamin A and thyroid function has been a frequent conclusion by clinicians and a few laboratory workers. The predominating opinion is that vitamin A is antagonistic to thyroid function. Too frequently, preparations containing large amounts of vitamin D have been employed in the laboratory as well as in the clinic. Drill has reviewed the subject up to 1943.[35] Publications since 1943 have not produced satisfactory evidence of either a direct action of vitamin A upon thyroid function or an indirect influence through the pituitary gland. Considerable evidence has appeared that there has been too little consideration and/or understanding of the proved consequence of vitamin A deficiency and of hypervitaminosis A.

2. Skeletal Responses[3, 4, 36]

As in vitamin A deficiency, the fully grown skeleton is not demonstrably influenced by excessive amounts administered to rats over long periods. In growing animals—rats, guinea pigs, mice, dogs, ducks, and chicks—profound changes result which can be correlated with the normal species growth patterns of the bones and accurately expressed as accelerations of those processes retarded by the deficiency.

In rats, guinea pigs, dogs, and chicks,[3, 4] the changes that have been studied histologically are those of epiphyseal cartilage and those concerning remodeling of bones—the resorption of bone, compact bone formation, and appositional bone formation. Common to all species is rapid maturation of epiphyseal cartilage cells and more rapid penetration by blood vessels. In guinea pigs the acceleration of endochondral bone formation, if the high vitamin A dosage (1000 to 1250 I.U. per gram) is begun a few days after birth, results in complete consumption of the cartilage in 10 to 15 days, and, as osteoid production keeps pace with the cartilage sequences, a plate of bone results as in the early stage of epiphyseal closure. This result, obtained in the distal femoral and proximal tibial epiphyses, corresponds to the normal at about 30 to 60 weeks of age, respectively (Fig. 36). Closure of the same epiphysis has not been the result in the rat, but in the normal animal closure of these epiphyses does not take place. Another species difference is that in the rat in experiments of short duration with heavy dosages the width of the epiphyseal cartilage plate may remain

[34] C. L. Maddock and S. B. Wolbach, *Federation Proc.* **9**, 337 (1950).
[35] V. A. Drill, *Physiol. Revs.* **23**, 355 (1943).
[36] T. E. Van Metre, Jr., *Bull. Johns Hopkins Hosp.* **81**, 305 (1947).

FIG. 36. Hypervitaminosis A in the guinea pig. Distal epiphyses of the femur showing early closure. All cartilage has disappeared. From 9 days of age this animal received approximately 600 I.U. of vitamin A per gram and 120 mg. of ascorbic acid daily for 18 days. The large amount of ascorbic acid did not influence the effect of the excessive vitamin A administration.

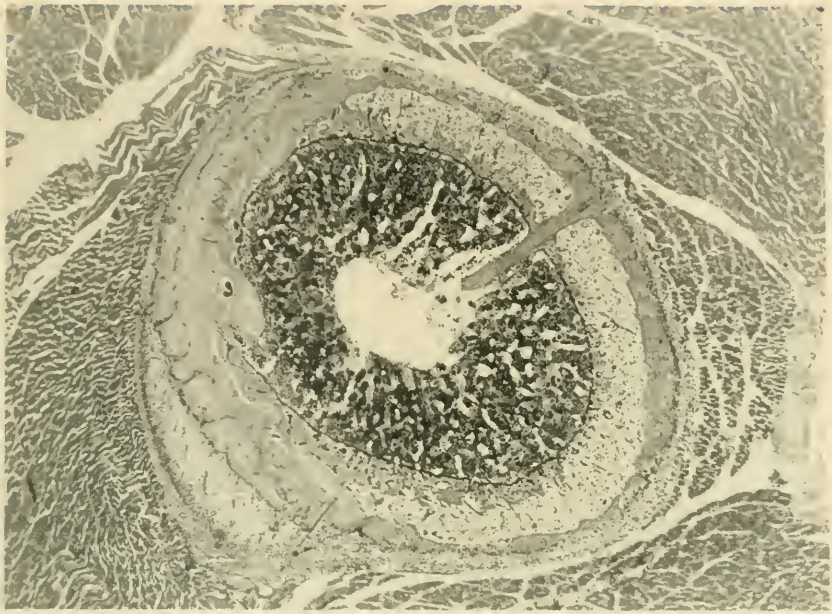

Fig. 37. Hypervitaminosis A in the rat. Result of 1250 I.U. per gram daily from the twenty-first to the twenty-eighth day of age. Cross section of the midportion of the femur. About one-third of the circumference of the cortex has undergone no appreciable change in conformity to the normal growth pattern. If a femur of a rat is rotated on its long axis there will come to view, on the postero-internal surface, a straight outline of about the middle third of the shaft.

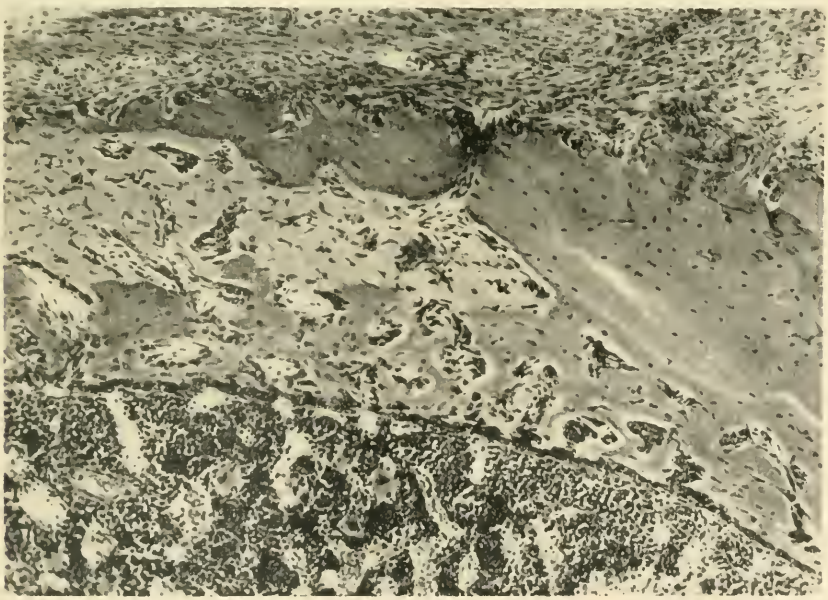

Fig. 38. Hypervitaminosis A in the rat. A high-power detail of Fig. 37, showing resorption of the external surface of the cortex and newly formed bone internally.

within the normal limits of variation, but the layers of growth and maturation are increased at the expense of the proliferative layer. Experiments of longer duration with lesser dosage reduced the epiphyseal cartilage to the state found at completion of growth. In the dog and the chick complete studies have not been made, although the same acceleration of epiphyseal cartilage sequences results. Concurrently with epiphyseal cartilage acceler-

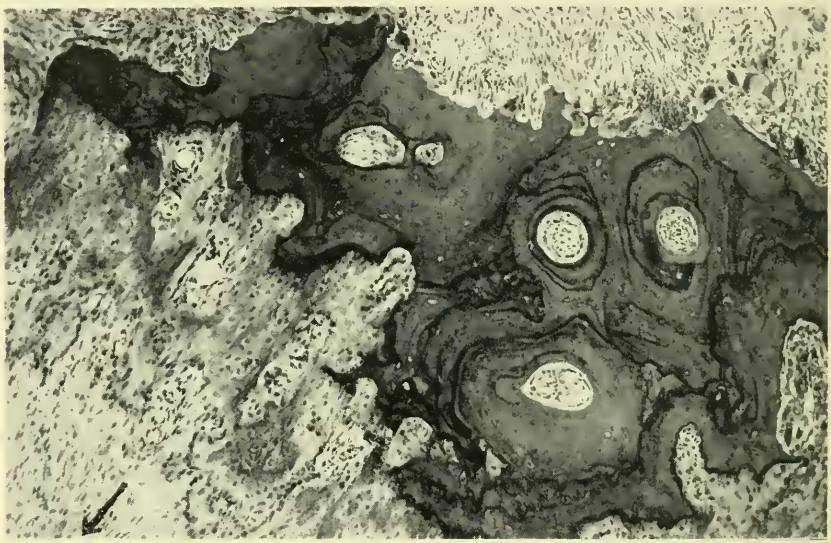

FIG. 39. Hypervitaminosis A in the dog. Cross section of the fibula from a puppy that had received 300 I.U. daily for 68 days from the age of 2 months. The arrow points in the direction of movement of the fibula as growth of the leg proceeds. The photomicrograph shows evidences of accelerated compact bone formation in the remaining fragment of old bone, many osteoclasts on the side undergoing resorption and much newly formed appositional bone on the opposite side. Cross sections of a fibula from a normal litter mate show the same pattern of resorption on one side and appositional bone growth on the side facing the direction of movement resulting from growth.

ation there is a great acceleration of the processes of resorption of bone and new bone formation essential to the remodeling that accompanies growth. This can be most easily followed at the ends of long bones where normal remodeling results in reduction of cross-sectional diameters and where it is achieved through resorption from without (periosteal surface) and new formation from within (endosteal). Resorption is by osteoclasis, and the histologic details are precisely those of normal osteoclasis. The new bone formation also is normal in histologic detail, as seen in appositional bone formation. The pattern exhibited in the accelerated remodeling corresponds precisely with that of normal growth, symmetrical or asymmetrical

as determined by the location and shown, respectively, at the proximal end of the tibia and the midshaft of the femur (Figs. 37 and 38).

The degree or rate of acceleration of these growth sequences varies with the amount of vitamin A administered and with the growth pattern of each bone. In the fibula in rat, guinea pig, and dog, because growth requires increasing separation from the tibia and a swing posteriorly, remodeling is normally rapid and clearly shown by cement lines. In hypervitaminosis A

FIG. 40. Hypervitaminosis A in the rat. Longitudinal section of a femur with a fracture near the distal epiphysis from a rat which had received 1250 I.U. of crystalline vitamin A per gram daily for 7 days following weaning at 21 days of age. The resorption of the exterior of the shaft and new formation of bone on the interior are apparent. There is considerable diminution of the width of the epiphyseal cartilage.

only traces of mature bone may remain at certain levels, while at the ends of the fibula where the normal rate of movement is slower, considerable mature bone remains (Fig. 39). The explanation of the fractures of long bones in rats, frequently erroneously ascribed either to "brittle bones" or to decalcification, is that, although new bone formation keeps pace with bone resorption, maturation of the newly formed bone matrix and its calcification exhibit the same lag as in normal bone growth, and hence the bone at sites of remodeling is structurally weak (Fig. 40). Fractures occurring during the deficiency are accompanied by voluminous callus formation. This applies to guinea pigs as well as to rats. Ascorbic acid up to 250 mg.

daily, given to 10-day-old guinea pigs during the period of excess vitamin A intake, does not modify the skeletal responses.

The study of hypervitaminosis A in the chick has covered only the first

FIG. 41. Hypervitaminosis A in the chick. Cross section of sacral vertebra through midportion of the rhomboidal fossa and the glycogen body. To illustrate the compact bone formation characteristic of hypervitaminosis A in this species and almost complete absence of epiphyseal cartilage. Compare with Fig. 42, showing the same region of a vitamin A-deficient chick of the same age, and Fig. 43, showing a vertebra at a higher level from an age control chick which shows the normal texture of the bones of the vertebrae.

32 days after hatching, a period in which the replacement of cartilage in the long bones is far from complete. Acceleration of endochondral bone formation and earlier establishment of epiphyseal cartilage plates are results. The pattern of remodeling sequence changes awaits knowledge of the normal. Accelerated compact bone formation in the chick is outstandingly more active than in mammals. It occurs in all bones but is most strikingy

shown in vertebrae and bones of the skull, including those of replacement origin (Figs. 41, 42, and 43).

In children and infants bone changes, thus far studied only by x-ray, are associated with painful swellings over long bones. All the numerous patients studied had received fish liver oil concentrates containing a large

FIG. 42. Vitamin A deficiency in the chick. Vertebra, midportion of rhomboidal fossa. Note inactive epiphyseal cartilages and character of the cancellous bone.

amount of vitamin D, which must be taken into account in consideration of the fact that the outstanding finding has been midshaft cortical hyperostoses of long bones, not correlatable with normal growth patterns and roentgenologically similar to "infantile cortical hyperostosis" of unknown etiology. Perhaps correlatable with normal growth are two other roentgenological findings, early formation of centers of ossification in advance of chronological age,[37] and irregular outlines of epiphyses,[38] both not conspicuous.[39, 40]

[37] H. W. Josephs, *Am. J. Diseases Children* **67**, 33 (1944).

[38] C. T. Friel and M. J. H. Grand, *Am. J. Diseases Children* **79**, 475 (1950).

In no reported human case did daily ingestion of vitamin A approximate the amounts used in the animal experiments described, and several to many months were required to elicit "toxic" signs of hypervitaminosis. Accumulative storage of vitamin A in the liver and the effects of excessive

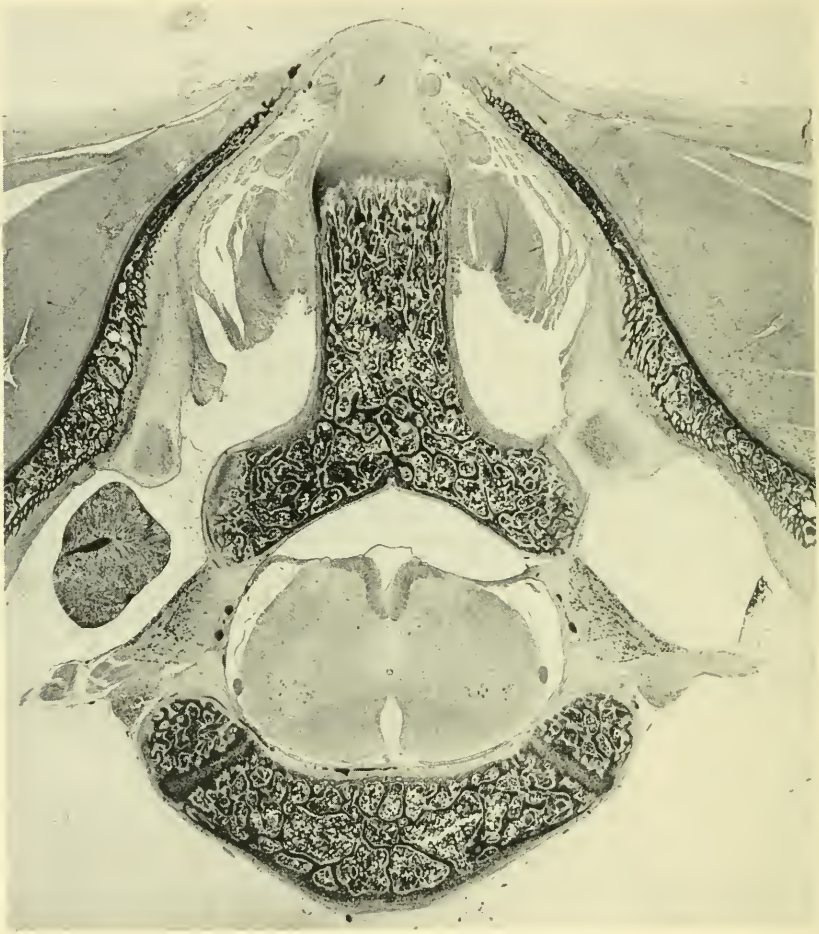

FIG. 43. Normal chick vertebral bone.

intake of vitamin D and possibly other substances in the fish liver oil concentrates require consideration in appraisal of current meager information on the pathology of hypervitaminosis A in humans.

Two, as yet inexplicable, effects of vitamin A upon bone removed from

[39] J. Caffey, *Am. J. Roentgenol. Radium Therapy* **65**, 12 (1951).
[40] D. Gribetz, S. H. Silverman, and A. E. Sobel, *Pediatries* **7**, 372 (1951).

the animal should be mentioned. Fell and Mellanby[41] placed tibiae, fibulae, radii, and ulnae of 17- to 21-day-old fetal mice into tissue culture media containing 1000 to 3000 I.U. of pure vitamin A acetate per 100 ml. of medium with the result that "the matrix of the terminal cartilage softened, shrank, and finally almost or completely disappeared, though the cartilage cells appeared normal; in the shaft the cartilage was rapidly replaced by marrow tissue; the bone was resorbed, sometimes completely; the soft tissue surrounding the explant grew as vigorously as in the controls cultivated in normal medium."

Barnicot[42] attached fragments of crystalline vitamin A acetate by means of fibrin to small pieces of parietal bone removed from 10-day-old mice and inserted the combination into the cerebral hemisphere of litter mates. After the expiration of 14 days, the site of the vitamin A acetate showed well-marked resorption and even perforation of the implanted bone. Osteoclasts, thought to be formed *de novo* in the vicinity of the implant, were regarded as the agents responsible for the resorption effect. Osteoblastic activities persisted in regions remote from the vitamin A acetate.

3. Teeth

The bone supporting the teeth exhibits accelerated growth sequences most prominent in the symphysis of the mandible. In guinea pig experiments with large dosages, the changes at the symphysis result in painful mobility. The growth sequences of the incisor teeth of rats and guinea pigs do not involve remodeling processes. The formation of dentine and enamel, though possibly accelerated, exhibits no apparent departure from the normal. Adequate studies of the effect of excess vitamin A on tooth growth have not been made.

4. Epithelial Tissues and Organs

Epithelia which undergo keratinizing metaplasia in vitamin A deficiency exhibit no change in hypervitaminosis A.

The gross skin manifestations in humans—adults, children, and infants—previously mentioned, have not been studied histologically, nor has their specificity for hypervitaminosis A been proved. As they also occur in other conditions, notably certain vitamin deficiency states, they may be the result of nonspecific nutritional disturbances. Lesions of an inflammatory nature in humans and experimental animals are found in those regions where vitamin A comes into direct contact with the skin—mouth parts of the human, and snout, paws, and other skin regions in animals. The gross and histologic changes described in the literature have been produced in this

[41] H. B. Fell and E. Mellanby, *Brit. Med. J. II*, 535 (1950).
[42] N. A. Barnicot, *J. Anat.* **84**, Part 4, 374 (1950).

laboratory by direct application of vitamin A solutions to the skin of mice, and fully described and illustrated for the rat in a recent paper.[43] No effect upon the skin of animals can be attributed to the internal absorption of vitamin A other than those common to severe nutritional disturbances in general.

Of the internal organs, the adrenals exhibit changes, moderate in the rat, conspicuous in the guinea pig. In both species the adrenal cortex enlarges. There is loss of cortical lipids. The number of mitoses in the facsicular zone greatly increases. In the guinea pig the glomerular zone atrophies and mitosis in the fascicular zone often remains incomplete, with dispersion and fragmentation of the chromosomes. Identical changes in the adrenals, however, are a feature of experimental scurvy in the guinea pig and of underfeeding in rats and guinea pigs.[24, 25] It is possible, however, that studies of the cortical lipids may reveal differences. Administration of 250 mg. of vitamin C given daily with the excessive amounts of vitamin A do not affect these adrenal gland changes in the guinea pig.

The other endocrine glands of special interest, because of clinical reports, are thyroid and hypophysis. A careful review of these glands from many rats and guinea pigs, all of which had pronounced skeletal effects, revealed no constant histological change when compared with normal controls. The anterior pituitary gland remained consistently normal in appearance in sections stained by the eosin-methylene blue technique. The thyroid glands varied in colloid content, vacuolization, staining, and height of alveolar epithelium to about the same degree found in normal controls. No evidence of inhibition of colloid secretion could be found. Even when compared with thyroids from vitamin A-deficient animals, no conclusions were possible from the histology that vitamin A has direct influence upon the thyroid. A similar study of parathyroid glands from the same animals was unrewarding, and the conclusion was unavoidable that this gland is not primarily influenced by vitamin A.

The lungs, the gastrointestinal tract, the pancreas—including the islands of Langerhans—and the genitourinary system of both sexes exhibited no effect of the hypervitaminosis.

The liver occasionally showed minute necroses and usually loss of fat vacuolization. Swollen endothelial cells of the sinusoids are the rule and have been shown to be the result of excess vitamin A storage.[44]

The spleen in all species showed some degree of hemosiderosis and fre-

[43] J. D. Sabella, H. A. Bern, and R. H. Kahn, *Proc. Soc. Exptl. Biol. Med.* **76**, 499 (1951).

[44] H. Popper, *Physiol. Revs.* **24**, 205 (1944).

quently thrombi in the sinuses of clumped red cell origin.[4] Lymph nodes of all species, usually from some one or several locations, contained red blood corpuscles in their sinuses, most markedly so in regions adjacent to hemorrhages.

The bone marrow in all species showed no constant change or evidence of specific vitamin A influence. Vascular lesions in arteries and veins were found in one of two dogs[4] and in a few of many rats studied, but whether or not these lesions are related to the hypoprothrombinemia associated with excess vitamin A remains hypothetical.

D. PRENATAL CONSEQUENCES OF VITAMIN A DEFICIENCY

Severe maternal vitamin A deficiency in pigs and rats results in death and resorption of the fetus. Both maternal and placental epithelia degenerate.[45] If the deficiency is not complete, the fetus may go to term and show abnormalities which can be related to arrestment of the skeleton and various organs. The first studies in this field were by Hale upon pigs.[46] Five-month-old gilts were kept on a vitamin A-deficient diet until signs of the deficiency were apparent (160 to 192 days). They were then bred to normal sires and kept on the deficient diet for 30 days, after which cod liver oil was given in order to permit completion of gestation. Otherwise, death of the fetuses resulted. Fetuses carried to term showed a variety of defects, including various stages of arrestment of formation of the eyes to complete lack of eyeballs, harelip, cleft palate, misplaced kidneys, and extra ear-like growths. Warkany[47, 48] in rats obtained arrestment of development of eyes, pleural cavities, lungs, heart, kidneys, testes, and diaphragm. Anomalies of the heart and blood vessels, all correlatable to arrestment of development at various embryological periods and similar to cardiovascular abnormalities of humans, have been described in the young of vitamin A-deficient rats.[49, 50]

An interesting result of maternal vitamin A deficiency in the rat is that described by Andersen,[51] who discovered that the expression of a "genetic trait"—congenital diaphragmatic hernia—in an inbred stock of albino rats could be enhanced (incidence 18.9%) or depressed (incidence of 0.9%) by

[45] K. E. Mason, *Am. J. Anat.* **57**, 303 (1935).
[46] F. Hale, *Am. J. Ophthalmol.* **18**, 1087 (1935).
[47] J. Warkany, *Vitamins and Hormones* **3**, 73 (1945).
[48] J. Warkany and C. B. Roth, *J. Nutrition* **35**, 1 (1948).
[49] J. G. Wilson and S. Barch, *Proc. Soc. Exptl. Biol. Med.* **72**, 687 (1949).
[50] J. G. Wilson and J. Warkany, *Pediatrics* **5**, 708 (1950).
[51] D. H. Andersen, *Am. J. Pathol.* **25**, 163 (1949).

the absence or presence of vitamin A in the diet. Although it has long been realized that precipitating factors in addition to hereditary factors combine in etiology of certain postnatal diseases (diabetes, pernicious anemia), this is the first instance where an antenatal precipitating factor has been demonstrated.

In view of the specific arrestment of postnatal replacement bone growth and the atrophy and keratinizing metaplasia of many epitheliums at all ages resulting from vitamin A deficiency, the consequences of the maternal deficiency upon the development of the fetus are not remarkable. Further experimentation is necessary to correlate congenital defects with degrees and timing of the maternal deficiency and with fetal developmental sequences. Such experimentation would further the study of the role of human maternal deficiencies in the causation of congenital malformations.

E. SUMMARY

The morphologic consequences of deficiency and excess of vitamin A place this substance in unique position among the vitamins. It is the only vitamin which, given in amounts in great excess over normal physiologic requirements, produces acceleration of a growth process (skeletal) unrelated to the growth of the organism as a whole. It is necessary for growth and differentiation of epiphyseal cartilage and for the maintenance of differentiation of many epithelia but not for their growth.

Its role in vision has been beautifully and completely elucidated, but otherwise its *in vivo* chemistry is unknown.

In spite of the behaviors suggestive of hormone-like properties, there is no evidence that it acts through an endocrine intermediary and there is considerable histologic evidence that it does not. It has been proved in this laboratory that the acceleration of skeletal growth sequences in hypervitaminosis A is unaffected in hypophysectomized rats. Similar proof is perhaps necessary for the exclusion of other endocrine glands as intermediaries. Impressive evidence that vitamin A acts directly upon competent tissues is furnished by experiments of Fell and Mellanby.[52] The cultivation of the ectoderm of chick embryos in media containing excessive vitamin A resulted in suppression of the formation of keratinizing epithelia and the substitution of a mucus-secreting ciliated epithelium. Explants into a normal medium resulted in differentiation of the basal cells into squamous keratinizing epithelium.

Results as described make obvious some possibilities of vitamin A experimentation for the anatomist, the embryologist, and, above all, the

[52] H. B. Fell and E. Mellanby, *J. Physiol.* **119**, 470 (1952)].

cytologist who, through application and extension of cytochemical methods, may blaze trails for the biochemist and geneticist.

VIII. Effects of Vitamin A Deficiency in Human Beings

KARL E. MASON

A. GENERAL CONSIDERATIONS

1. "Primary" and "Secondary" Deficiency

Vitamin A deficiency, or avitaminosis A, is termed "primary" when it results from an inadequacy of vitamin A or its precursor, carotene, in the diet. It is called "secondary" or "conditioned" when it occurs as a result of (1) disorders which interfere with the absorption or storage of the vitamin or provitamin, as represented by celiac disease, cystic fibrosis of the pancreas, sprue, giardiasis, congenital absence or obstruction of the bile duct, cirrhosis of the liver, ulcerative colitis, and prolonged, severe diarrhea; (2) disorders interfering with the conversion of carotene to vitamin A, such as may occur in diabetes mellitus and hypothyroidism; and (3) factors causing unusually rapid utilization or loss of vitamin A in the body, such as acute or chronic infections with associated high and sustained fever. These conditioning factors exert their effect not so much by depleting body stores of vitamin A, of which 90 to 95 % is represented by liver storage, as by diminishing vitamin A levels in the circulating plasma, and consequently in body tissues generally, either by inhibiting its release from storage depots, by depressing the vitamin A carrying capacity of the plasma, or by inactivating vitamin A after its release into the circulation. States of "conditioned" deficiency rarely lead to outspoken manifestations of avitaminosis A. They may, however, account for variations in incidence and severity of manifestations of "primary" vitamin A deficiency in groups or populations existing on marginal levels of vitamin A intake, and also for differences in responses to comparable levels of vitamin A therapy.

The vitamin A storage in well-nourished individuals is of such magnitude, and the vitamin so tenaciously held by tissues, that at least 2 to 3 years of severe deprivation would be required to bring about clear-cut deficiency symptoms and pathologic lesions. For this reason, avitaminosis A occurs but rarely in adult man and usually in association with a very prolonged intake of generally inadequate and unbalanced diets deficient to a variable degree in dietary essentials other than vitamin A; in other words, avitaminosis A is usually a more or less predominant phase of a "multiple deficiency" state, except when induced in human volunteers by careful dietetic manage-

ment. In the case of infants and young children, where prior storage of the vitamin is more limited, outright vitamin A deficiency is more common but is not often a simple deficiency state. Much of what we know regarding symptoms and pathologic changes in tissues resulting from vitamin A deficiency in human beings is based upon clinical observations on infants and young children.

Uncomplicated vitamin A deficiency in laboratory animals leads to three primary dysfunctions: (1) defective vision in dim light, due to needs for vitamin A as a precursor of visual purple in rod cells of the retina; (2) atrophy and keratinizing metaplasia of many epithelia of the body; and (3) disturbances in the appositional growth and remodeling of bone. These dysfunctions and lesions are most readily induced by deprivation of vitamin A and carotene during early life and require much more prolonged periods of depletion in older animals with appreciable body stores of the vitamins. In fact, alterations of bone growth occur only in young, rapidly growing animals and have been demonstrated only in laboratory animals. This leaves for our consideration the visual dysfunctions, which are primarily physiologic, and the epithelial alterations, which represent the major histopathologic manifestations of the deficiency state. Before discussing the nature of these phenomena in man, it may be pertinent to consider the types and sources of evidence on which the clinical findings, and our ultimate conclusions, are based.

2. Deficiency in Human Volunteers

One approach to an evaluation of the effects of vitamin A depletion in man is the maintenance of human volunteers on a diet deficient in vitamin A but otherwise adequate until the appearance of specific symptoms which can be carefully studied and then shown to disappear following vitamin A therapy. Even then, information regarding pathologic changes in tissues is limited to material obtained through biopsy. Between the years 1937 and 1943 eight experiments of this type were reported, largely in an effort to evaluate photometric measurements of dark dysadaptation (night blindness) as a criterion of suboptimal intake of vitamin A. The depletion periods varied from 1 to 7½ months. The results were far from conclusive, and indicated the need for a more exacting investigation extending over a much longer period.

A study of this type was undertaken in 1942 at the Sorbey Research Institute in Sheffield, England, because of need for more accurate data on human requirements for vitamin A.[1] Twenty-three conscientious objectors

[1] E. M. Hume and H. A. Krebs, Vitamin A Requirement of Human Adults; An Experimental Study of Vitamin A Deprivation in Man (A Report of the Vitamin A Sub-Committee of the Accessory Food Factors Committee), *Med. Research Council (Brit.) Spec. Rept. Ser.* **264**, (1949).

lived on a diet essentially devoid of vitamin A and providing little more than traces of β-carotene. Although several subjects were depleted for more than 1½ years, and 1 subject for more than 2 years, there was no more than slight evidence of deficiency manifestations in 3 subjects, based upon diminished plasma vitamin A levels and slight impairment of dark adaptation. This herculean effort emphasizes the capacity of the healthy adult to retain and utilize stores of vitamin A previously acquired by the body. It also confirms previous observations that the first effect of vitamin A depletion is a prompt and pronounced decrease in carotenoid content of the blood plasma. On the other hand, it required about 8 months of depletion before an appreciable diminution in plasma vitamin A levels occurred, and 14 months or more before levels of less than 40 I.U. per 100 ml., generally regarded as a critical level, were observed in 3 subjects.

3. Nutrition Surveys

Representative individuals, or representative families, of a community may be assessed on the basis of: (1) the daily intake of vitamin A and of carotene provided by their average diet; (2) the incidence of recognized symptoms of vitamin A deficiency (e.g., night blindness, xerophthalmia, keratomalacia, skin lesions); and (3) plasma levels of vitamin A. Unfortunately, avitaminosis A in man is often difficult to diagnose and to evaluate qualitatively. Much uncertainty still exists as to the reliability of methods for measuring night blindness, which are difficult to apply in field surveys; and there is lack of accord among investigators concerning the specificity of conjunctival and cutaneous lesions and their differentiation from similar lesions due to other causes. These criteria have increased significance if there is opportunity to test their response to vitamin A therapy. Serum vitamin A levels have definite meaning when below certain critical levels, but they seem not to afford a reliable criterion of subclinical deficiency. Data obtained by survey studies provide useful information regarding incidence of deficiency and possible merits of diagnostic criteria used but usually add little to our knowledge of the pathologic features of the deficiency manifestations.

4. Clinicopathologic Studies

Symptoms or manifestations suggestive of inadequacy of vitamin A occasionally are noted in routine examination of patients attending large outpatient clinics or in nutritional surveys. Where the clinician supplements this evidence with other data designed either to eliminate or to confirm the suspected implication of vitamin A (e.g., dietary history, evaluation of dark adaptation, plasma levels of vitamin A, biopsy and microscopic study of tissues, conditioning factors, response to vitamin A therapy), important contributions can be made to an understanding of pathologic manifesta-

tions of the deficiency disease. It might be added, however, that there exists no morphologic alteration of tissue which alone can be considered strictly pathognomonic of vitamin A deficiency. Where the clinician is content merely to prescribe an increased intake of vitamin A, and to attribute any observed remission of symptoms to this therapeutic measure, an uncertain and often contradictory body of evidence accumulates. Unfortunately the latter type of evidence is often too prevalent in the clinical literature.

5. Vitamin A and Epithelia

The most striking histopathologic manifestation of vitamin A depletion, as observed in a variety of experimental animals and in man, is the inability to maintain the morphologic integrity of various specialized epithelia. Epithelia which are normally stratified and keratinized, such as the epidermis proper, are thought to undergo excessive keratinization of the superficial layers (hyperkeratosis). In epithelia which undergo only periodic keratinization, such as the vaginal epithelium of rodents during estrus, a state of continuous cornification results. In epithelia of the stratified squamous type covering mucous surfaces such as those of the cornea and conjunctivae, and in transitional epithelia such as are found in the renal pelvis and bladder, the superficial cells are replaced by layers of keratinized cells. The columnar or cuboidal cells lining ducts of glands often become flattened and cornified. More highly differentiated epithelia, such as the pseudostratified columnar ciliated of the respiratory tract (nares, paranasal sinuses, larynx, trachea, and bronchi), respond to vitamin A depletion by atrophy of the superficial cell layers which are replaced by a stratified keratinized type of epithelium. The germinal epithelium of the testes and the enamel organ of the tooth become atrophic but not keratinized. Mucous and mucoserous glands related to the involved mucous surfaces become hypofunctional and atrophic. Certain glands, especially those of ectodermal origin (sebaceous, sweat, lacrimal) may exhibit diminished functional activity; actual atrophy of the gland parenchyma may occur. It is often difficult to determine whether this atrophy represents a direct effect or is secondary to duct blockage by keratinized cells, a process frequently observed in ducts of glands in which alterations of parenchymal structure are not observed.

The general picture is one of a deep-seated disturbance in basal cells of various types of epithelia which are unable to differentiate in the manner normally typical for each location and can give rise only to cells with potentialities for producing a stratified keratinized type of epithelium. There is thus a change from one type of epithelium to another, or a metaplasia, always directed toward a stratified keratinized layer; hence the term

"keratinizing metaplasia," frequently used to describe the reaction of the epithelial surfaces. This may be looked upon as the easiest type of structural pattern for the basal cells to produce and maintain; a reaction to adverse conditions comparable, in a sense, to encystment in lower forms of life.

Despite the striking alterations often observed, vitamin A therapy eventually results in complete restoration of the epithelium previously existing at the involved surface, provided that repair is not complicated by tissue damage resulting from localized infections and inflammatory reactions. Of particular interest is the fact that cells of the stratum germinativum of the metaplastic epithelium preserve, for an indefinite period, the potentiality to differentiate and fulfill their destiny whenever an adequacy of vitamin A is acquired by the organism.

B. NIGHT BLINDNESS

Recognized in Egyptian medicine as a disorder of the eyes curable by ingestion of animal livers, night blindness represents one of the oldest diseases known to man and the first nutritional disorder for which an effective therapy was established. There are many records of its sporadic appearance in populations existing for long periods on severely restricted diets, especially as the result of famine or wars. It is regarded as one of the most reliable signs of low vitamin A status in man. Since it is not easy to detect in very young infants and appears so insidiously in older children and adults, it has often been overlooked or ignored until the appearance of xerophthalmia and keratomalacia.

According to their etymology, the terms "nyctalopia" and "hemeralopia" refer to impaired vision in dim light (scotopic or rod vision) and in bright light (photopic or cone vision), respectively. However, from ancient times to the present day each term has been used in either sense, and general usage has favored the term hemeralopia (though, strictly-speaking, the incorrect term) as synonymous with night blindness. It may also be pertinent to note here that there is growing evidence that vitamin A plays an important role in cone vision as well as a predominant role in rod vision.

1. Vitamin A in the Visual Purple Cycle

This impairment of vision in dim light manifests itself as sensitivity to bright light, difficulty in reading unless the light is brilliant, glittering images and dancing specks before the eyes, tendency to stumble or to bump into objects in dim light, and prolonged delay in adaptation from bright to dim light. These defects are much more marked in evening twilight than in the dim light of daybreak. Visual adaptation to dim light depends upon the presence of adequate amounts of visual purple (rhodop-

sin) in the retinal rod cells. The impact of light upon the retinal rods initiates a reversible process involving the breakdown of rhodopsin (visual purple) to a yellow compound of retinene (vitamin A aldehyde) and opsin (a specific protein of the retina), and subsequent spontaneous resynthesis of rhodopsin from these two substances. However, during this cycle some of the retinene breaks away from its combination with opsin and decomposes to colorless vitamin A and to compounds without vitamin A activity. This necessitates reformation from stores of vitamin A in the pigmented epithelium of the retina, the blood, and body tissues generally. When the latter are deficient, resynthesis of visual purple is delayed and is reflected in impaired visual adaptation of the individual to dim light. A recent report by Wald[2] presents evidence that enzymatic reactions in the visual purple cycle involve a retinene reductase system in which cozymase (DPN) acts as a coenzyme. Since nicotinamide, the antipellagra factor, is an essential component of cozymase, a second vitamin is implicated in this visual process. Wald also states that alcohol dehydrogenase from liver can substitute for the retinene reductase of retinal extracts. This suggests that further clarification of these enzymatic reactions may provide the key to the basic functions of vitamin A in epithelial cells in general and the underlying causes of keratinizing metaplasia.

2. Measurement of Night Blindness

There is little doubt that impaired dark adaptation represents the earliest physiologic manifestation of vitamin A deficiency. However, since febrile states, generalized infections, and acute illnesses may also impair rod vision, it is not pathognomonic of the deficiency state. Evaluation of dark adaptation by various types of adaptometers and biophotometers has led to rather discordant results. These appear to be due in part to differences inherent in the physical instruments and in their use, and in part to variations among individuals in their metabolic utilization and storage of vitamin A.[3] When these instruments are effectively applied, it seems that a good measure of impaired dark adaptation is obtained, except in instances where the visual defect is minimal.[4, 5] The time-consuming nature of the test, combined with the apparatus and trained personnel required, limits the usefulness of the method as a diagnostic aid.

C. XEROPHTHALMIA AND KERATOMALACIA

Xerophthalmia refers to a dry, roughened state (xerosis) of the conjunctivae and cornea, often associated with the occurrence of irregular whitish

[2] G. Wald, *Science* **113,** 287 (1951).

[3] J. J. Stern, Nutrition in Ophthalmology, Nutrition Monograph Series, No. 1, National Vitamin Foundation, New York, (1950).

[4] P. C. Jeans, E. L. Blanchard, and F. E. Satterthwaite, *J. Pediat.* **18,** 170 (1941).

[5] R. E. Eckardt and L. V. Johnson, *J. Pediat.* **18,** 195 (1941).

placques (Bitot's spots) on the scleral conjunctiva. Keratomalacia refers to later manifestations of the same processes, involving edema, infiltration, and necrotic softening of the cornea, which may be followed by perforation, hypopyon, and panophthalmitis.

1. Early Investigations

These ophthalmic disorders were well recognized by European clinicians for almost 100 years before they became established as symptoms of vitamin A deficiency. During the latter half of the nineteenth century they were reported from all parts of the world, their frequent association with night blindness noted, and the beneficial effect of liver and of cod liver oil recorded. Bitot's[6] description (1863) of the placques which bear his name was based upon 29 patients, 9 to 19 years of age, all of whom showed night blindness. As early as 1876, Snell[7] was successfully treating cases of night blindness with Bitot's spots with cod liver oil and steel (a compound of iron salts) as if that were the generally accepted remedy.

The first account of the pathologic anatomy of the lesions, emphasizing hyperplasia of the corneoconjunctival mucosa with marked flattening of the superficial epithelium, was presented by Leber[8] in 1883. In that same year, de Gouvea[9] described a series of cases in plantation slaves in Rio de Janerio, and reported effective treatment with cod liver oil. Macroscopical changes only were emphasized in the classic studies of Mori,[10] based on 1511 cases of xerophthalmia and 116 cases of keratomalacia observed in Japan during the years 1899 to 1903, and in those of Bloch[11] and Blegvad[12] which, combined, represented observations on 211 cases of xerophthalmia and 453 cases of keratomalacia observed from 1909 to 1920 in Denmark. Mori noted a high incidence in late winter (when vitamin A storage and intake are lowest) and again in association with summer diarrhea (interfering with absorption and utilization of vitamin A). Although he related the symptoms to lack of fats in the diet, he recognized that cod liver oil was much more effective therapeutically than vegetable oils. The Danish clinicians related the ocular disorders to restricted diets composed chiefly of skimmed milk and gruels, and the widespread exportation of butter during World War I with replacement by vegetable margerines. The discovery of vitamin A as a dietary essential at this same period, and the demonstration that similar eye lesions occur in laboratory animals deficient in this vitamin,

[6] P. Bitot, *Gazz. med. Paris* **18**, 435 (1863).
[7] S. Snell, *Lancet* **I**, 8 (1876).
[8] T. Leber, *Albrecht von Graefe's Arch. Ophthalmol.* **29**, (3), 225 (1883).
[9] H. de Gouvea, *Albrecht von Graefe's Arch. Ophthalmol.* **29**, (1), 167 (1883).
[10] M. Mori, *Jahrb. Kinderheilk.* **59**, 175 (1904).
[11] C. E. Bloch, *J. Hyg.* **19**, 283 (1921); *Am. J. Diseases Children* **27**, 139 (1924); **28**, 659 (1924).
[12] O. Blegvad, *Am. J. Ophthalmol.* **7**, 89 (1924).

clarified and established the etiology of xerophthalmia and keratomalacia. In the Japanese studies keratomalacia was relatively less common than in the Danish outbreaks. Since the Japanese children were breast-fed and derived limited amounts of vitamin A from the mother's milk, symptoms appeared chiefly during the postweaning period (2 to 5 years of age). The Danish children were mostly bottle-fed with skimmed cow's milk, often boiled with consequent loss of vitamin A; hence, the symptoms appeared earlier and in a more acute form. It is well recognized that, the younger the infant, the more rapidly the ocular symptoms develop, and that in very young infants signs of xerophthalmia and keratomalacia appear in rapid sequence. In fact, keratomalacia has been observed in breast-fed infants[13, 14] and also in the newborn infant.[15] The incidence of xerophthalmia is low in adults and particularly high in infants and young, actively growing children. Some investigators consider the cornea of infants especially vulnerable.

2. External Manifestations

The usual sequence of ocular changes, as seen on ophthalmologic examination, is as follows: (1) night blindness; (2) dryness of the conjunctivae and diminution in lacrimal secretions; (3) loss of normal luster of conjunctival surfaces; (4) thickening, roughening, and wrinkling of the bulbar conjunctiva; (5) appearance of foam-like, irregular placques on the sclera of the palpebral fissure (Bitot's spots), and similar xerotic patches on the subpalpebral sclera; (6) increasing opaqueness and hypoesthesia of the sclera and cornea; (7) impaired vision in bright light; (8) edema, photophobia, leucocytic infiltration, and necrotic softening of the cornea (stage of keratomalacia); (9) marked corneal opacity, corneal perforation, infection of the anterior chamber, prolapse of the iris, and panophthalmitis. Body temperature is said to be increased in adults[16] and children[17] with keratomalacia, returning to normal when other symptoms have regressed following therapy.

The palpebral conjunctiva, perhaps because of its more protected location, rarely shows more than a yellowish reddening, with some granulation and thickening in more advanced stages of the ocular lesions; the conjunctiva of the fornix and adjacent sclera usually shows yellowish to brownish-grey discoloration in dark-skinned races.[16] Smears of epithelial scrapings from the conjunctivae always contain a great abundance of bacterial organisms of a variety of types. These are considered to have no specific relation-

[13] J. Thalberg, *Arch. Augenheilk.* **12,** 314 (1883).
[14] J. H. deHaas, J. H. Posthuma, and O. Meulemans, *Indian J. Pediat.* **8,** 139 (1941).
[15] J. P. Maxwell, *J. Obstet. Gynaecol. Brit. Empire* **39,** 764 (1932).
[16] A. Pillat, *Arch. Ophthalmol. (Chicago)* **2,** 256, 399 (1929).
[17] K. L. Hsu, *China Med. J.* **41,** 825 (1927).

ship to the pathologic changes but probably represent organisms normally present in the conjunctival sac which thrive in greater numbers in the debris of sloughed epithelial cells.

Kruse[18] has described the widespread occurrence of flat or slightly raised, whitish to yellow conjunctival spots, as seen under biomicroscopic examination of the eye. Unlike Bitot's spots, these result from cellular infiltration and other alterations in the subepithelial tissues. Kruse thinks that they may reflect a prolonged but mild insufficiency of vitamin A. The validity of these interpretations has been challenged by others, especially Berliner,[18a] who regards these spots as pingueculae, although Kruse[18b] has ably defended his beliefs. The varied evidence on this question, recently summarized by Stern,[3] is still insufficient to permit a satisfactory conclusion as to the diagnostic value of these conjunctival changes.

3. Histopathology

Descriptions of the histopathologic changes characterizing xerophthalmia and keratomalacia in man are meager. Fatal outcome is rare and occurs usually in infants as a result of associated pneumonic disease. Permission for removal of orbital structures at necropsy is not easy to obtain. Since ophthalmic examination provides much information concerning the superficial changes, and since much attention has been given to the histopathology in experimental animals, there has been only limited exploration of the pathologic changes in man.

As early as 1883, Leber[8] described a thickening and hyperplasia of the bulbar conjunctiva, with marked flattening and cornification of the superficial cells; a similar change was also noted in the mucosa of the renal pelvis. Little has been added since that time except for the studies of Kreiker[19] and Sweet and K'ang,[20] based on material obtained by biopsy and at necropsy. Their studies indicate that the earliest morphologic change is a disappearance of goblet mucous cells from the epithelium of the bulbar conjunctiva and cornea, followed by hyaline-like changes in the superficial cells (stage of prexerosis). These are soon undermined and lifted off by flattened, keratohyalin-laden cells of the deeper layers which progressively cornify. Eventually there is formed a somewhat hyperplastic, keratinized stratified epithelium resembling that of the epidermis (stage of xerosis conjunctiva). In dark-skinned races considerable melanin pigment accumulates in the basal cells of the conjunctival epithelium,[16, 21] which is respon-

[18] H. D. Kruse, *Milbank Mem. Fund Quart.* **19**, 207 (1941).
[18a] M. L. Berliner, *Am. J. Ophthalmol.* **50**, 302 (1942).
[18b] H. D. Kruse, *Am. J. Ophthalmol.* **50**, 1386 (1942).
[19] A. Kreiker, *Albrecht von Graefe's Arch. Ophthalmol.* **124**, 190 (1930).
[20] L. K. Sweet and H. J. K'ang, *Am. J. Diseases Children* **50**, 699 (1935).
[21] A. Pillat, *Arch. Ophthalmol. (Chicago)* **9**, 25 (1933).

sible for the greyish-yellow discoloration especially apparent grossly in the conjunctivae of the fornices and semilunar fold; there may also be migration of chromatophores from the limbal pigment ring, giving rise to a smoky discoloration of the bulbar conjunctiva.

Bitot's spots represent one of the most characteristic features of well-established xerophthalmia. They consist of sharply defined, rounded, triangular (with base toward the corneal limbus), or striated and band-like, whitish, foam-like patches located on the sclera of the temporal (usually) palpebral fissure. Fasal[22] provides a good picture of these spots. These areas are not wetted by tears, and consist of accumulations of epithelial debris, sebum from Meibomian glands, fatty globules, and masses of xerosis bacilli and other organisms. They detach readily and are rarely seen in histologic sections of the eyeball.[19] When scraped away they reform rapidly. Patchy islands of xerosis resembling Bitot's spots, often coalescing into much larger placques, may occur on the more protected surfaces of the sclera, in more advanced stages.[16] It is worthy of note that a chronic type of Bitot's spot, not associated with low vitamin A blood levels or other evidence of the deficiency state and not responding to vitamin A therapy, has been reported from Java[23] and Trinidad.[24]

The cornea shows increasing opaqueness and thickening; patchy areas of xerosis, resembling Bitot's spots, sometimes occur.[16] This is followed by exfoliation of the epithelial surface, bacterial invasion, thickening and edema of the stroma, infiltration of lymphoid cells and leucocytes, ingrowth of blood vessels and focal necrosis leading to ulceration, perforation, and other ocular pathology such as is observed after various types of corneal injury. A thickening of nerve fibers distributed to the cornea,[16] and pronounced loss of sensibility of corneal and scleral surfaces,[16, 25] have led to suggestions that the ocular lesions, and alterations of epithelia elsewhere, might be secondary to degenerative changes in sensory nerves and indicate a neurotrophic function of vitamin A. This concept has not been supported by careful experimental studies on rabbits,[26, 27] in which the ocular changes occurring in vitamin A deficiency and during therapeutic repair are quite comparable to those observed in man.

The histopathology of the lacrimal gland, palpebral conjunctiva, and glands of the eyelids has not been explored. On the basis of gross observations of the ocular apparatus, and pathologic changes observed in other epithelia, it would seem that the palpebral conjunctiva suffers no more

[22] P. Fasal, *Arch. Dermatol. and Syphilol.* **50**, 160 (1944).
[23] Sie-Boen-Lian, *Albrecht von Graefe's Arch. Ophthalmol.* **139**, 17 (1938).
[24] V. M. Metivier, *Am. J. Ophthalmol.* **24**, 1029 (1941).
[25] I. John, *Arch. Ophthalmol. (Chicago)* **5**, 374 (1931).
[26] M. V. Radhakrishna Rao, *Indian J. Med. Research* **24**, 439 (1936).
[27] I. A. Mann, A. Pirie, K. Tansley, and C. Wood, *Am. J. Ophthalmol.* **29**, 801 (1946).

than mild xerosis, and that the lacrimal and tarsal glands may manifest some degree of hypofunction but no morphologic change other than that which may be secondary to keratinizing metaplasia of their duct epithelium.

4. Response to Therapy

With adequate vitamin A therapy, there is disappearance of Bitot's spots within 2 weeks or less,[22, 28] and of other signs of xerophthalmia within 1 or 2 weeks thereafter; complete histologic restitution of all tissues involved may require as long as 2 months, though disappearance of excess pigment may require many months.

In keratomalacia there is a striking improvement in gross appearance of the eye during the first week of therapy, with rapid healing of ulcerated areas, but maximal repair usually requires many months. Pillat[16, 21] has given the most complete description of progressive stages of repair. Scars may be left in local areas of irreparable damage.

When corneal perforation, panophthalmitis, and prolapse of the iris have occurred, partial or complete impairment of vision results. It is stated that about one-fourth of cases of well-established keratomalacia which survive become totally blind, and about the same proportion lose vision in one eye; the latter is related to the fact that one eye is usually more involved than the other. In Occidental and Oriental countries keratomalacia has represented the most common cause of blindness. A recent report from Calcutta[29] refers to 150 cases seen in an out-patient clinic over a 6-month period, with a high incidence of night blindness and xerophthalmia, and states that "Vitamin A deficiency is a common disease amongst the children in this country. It heads the list of avitaminoses . . . ."

D. METAPLASIA OF EPITHELIA (INTERNAL)

Early clinicians frequently commented upon dryness of oral, nasal, and pharyngeal membranes, hoarseness, husky voice, and dry cough in patients with keratomalacia; also the frequency with which respiratory infections, chiefly pneumonia, were associated with acute phases of the disorders and constituted the primary cause of death in untreated or inadequately treated cases. Mention has been made of the areas of keratinization in the mucosa of the renal pelvis noted by Leber[8] in 1883. No other post-mortem studies on avitaminosis A were reported until 1922, when Wilson and DuBois[30] described widespread keratinizing metaplasia in epithelia of the respiratory tract and of ducts of various glands in a 5-month infant with keratomalacia. Some years later there followed reports on 1 case by Thatcher and Sure,[31]

[28] J. C. Spence, *Arch. Disease Childhood* **6**, 17 (1931).
[29] D. Pal, *Indian J. Pediat.* **16**, 1 (1949).
[30] J. R. Wilson, and R. O. DuBois, *Am. J. Diseases Children* **26**, 431 (1923).
[31] H. S. Thatcher and B. Sure, *Arch. Pathol.* **13**, 756 (1932).

11 cases by Blackfan and Wolbach[32] (including the case described by Wilson and DuBois), and 17 cases by Sweet and K'ang.[20]

1. Post-Mortem Material

These 29 post-mortems form the basis of our knowledge regarding the internal pathology of vitamin A deficiency in man. These studies suffer from the fact that in most cases vitamin A therapy had been instituted for variable periods (less than 1 week in about one-third, and between 1 and 5 weeks in more than one-half of the cases) so that varying amounts of epithelial repair had often occurred before exitus. However, 6 of the cases studied by Blackfan and Wolbach[32] were untreated cases of keratomalacia. The findings in the separate pathologic studies are fundamentally the same, and in general accord with those obtained in experimental studies.

Considering the 29 cases as a group, it is of interest that approximately two-thirds exhibited either xerophthalmia or keratomalacia, were less than 18 months of age, and succumbed as a result of bronchopneumonia. Three young adults (18, 23, and 32 years of age), on vitamin A therapy for 2 to 5 weeks,[20] were the only subjects over 4 years of age. On the whole, the extent of epithelial alteration was quite commensurate with the severity of deficiency manifestations and the extent of vitamin A therapy. Conclusions and deductions which may be made from the pathologic studies reported are as follows.

2. Epithelial Metaplasia

In general, keratinizing metaplasia of epithelia of internal surfaces of the body is a relatively late manifestation of avitaminosis A. Whether the susceptibility of these epithelia varies in relation to age has not been established. The metaplastic change may in some instances be quite widespread and in other cases restricted to localized patch-like areas.

The "respiratory epithelium," lining most of the respiratory system, seems especially prone to keratinizing metaplasia; it is also slower than other epithelia in effecting full morphologic restoration after vitamin A therapy. This is compatible with the highly specialized character of this pseudostratified columnar epithelium, with an abundance of ciliated and mucous cells and a surface bathed by secretions from many underlying mucous and mucoserous glands. The metaplasia of this epithelium constitutes the most impressive evidence of the role of vitamin A in maintaining the functional and structural integrity of specialized epithelia, and the particular susceptibility of mucus-secreting cells to lack of vitamin A. Keratinization and blockage of ducts may lead to dilation and hypofunction of the underlying glands. The adjacent mucosa is often the site of infiltra-

[32] K. D. Blackfan and S. B. Wolbach, *J. Pediat.* **3**, 679 (1933).

tion of lymphoid cells, mononuclears, and leucocytes. The lining of the nares, nasopharynx, larynx, trachea, bronchi, and bronchioles is involved in much the same manner but to varying degrees.

The transitional epithelium of the renal pelvis is much more often involved than is that of the ureter or urinary bladder. Metaplasia occurs at times in the epithelium of the uterus and prostate. In the digestive tract, it has been observed chiefly in ducts of the salivary glands (where obstruction may cause dilation and sometimes atrophy of the acini), esophageal lining, and ducts of esophageal glands. Keratinization of pancreatic ducts, cystic dilation of some acini, and varying degrees of fibrosis of the pancreas have been observed, but their specific relation to vitamin A deficiency is questionable.[32] Among other non-specific findings are atrophy of adipose tissue with reversion to the embryonic glandular type, hyaline degeneration of skeletal muscle fibers, arrest of bone growth and cartilage proliferation, and hemosiderosis in spleen and liver.

E. CUTANEOUS LESIONS

The malnourished subject presenting evidence of avitaminosis A is at best a variable and complex entity. Reactions of the integument, whether ascribed to lack of vitamin A or not, are subject to modification by environmental and metabolic influences which vary greatly with the age of the individual. Cutaneous lesions, especially those involving the pilosebaceous follicle, often present difficult problems in differential diagnosis. Furthermore, dermatologic terminology is not blessed with a high degree of uniformity or specificity. It is therefore not surprising that during the past twenty years there has appeared an extensive but rather controversial literature dealing with the character, specificity, and diagnostic value of cutaneous lesions which have been observed in association with other manifestations of avitaminosis A in man. A somewhat chronologic treatment of the evidence bearing on this subject provides perhaps the best means of reaching a proper appreciation and evaluation of the observations recorded.

1. Macroscopic Features in Adults

a. Studies in China

Although early accounts of xerophthalmia and keratomalacia contain occasional reference to dry and wrinkled skin, chiefly in children, the first detailed account of cutaneous changes related to avitaminosis A is contained in the report of Pillat[16] dealing primarily with keratomalacia in 6 adult patients studied in the hospital of Peiping Union Medical College during 1928. The hair was described as dull, dry, coarse, and brittle. The skin over the entire body except that of the face was generally desiccated, finely wrinkled, covered with fine scales, earthy brown to slate grey in

color, and branny in texture as if strewn with coarse powder. There was no evidence of sweating. Some patients showed chalky, opaque, and furrowed nails, a few had numerous comedones on the face, neck, and chest. Body temperature was frequently elevated and attributed to impaired heat-regulating mechanisms of the integument; a similar phenomenon has been noted in infants with keratomalacia[9, 17, 20] and ascribed to more deep-seated metabolic disturbances.[20] Pillat emphasized the widespread effects upon the ectodermal leaf of the body, but made no microscopic study of the skin; nor did he refer to follicular eruptions which were described two years later by Frazier and Hu[33] and have been generally accepted as specific manifestations of avitaminosis A.

Frazier and Hu based their observations on 15 patients, of which 14 were soldiers, studied in the same hospital during the following year. They further indicate[34] that these were from a group of 209 soldiers with ocular lesions which Pillat had examined, so that Pillat could not have been unaware of the follicular type of skin lesion. According to the history given by the patients, the follicular lesions had usually preceded the onset of keratomalacia, which was of 2 to 12 weeks' duration. Skin changes similar to, but less severe than, those described by Pillat were noted in most of these patients. Particular attention, however, was given to the papular eruption to which they[34] applied the term "follicular hyperkeratosis." Since their observations have served as the standard of reference against which subsequent findings have been compared, it seems appropriate to quote their original description of the lesions:[33]

> Several weeks prior to the onset of the ocular symptoms, the skin became dry and slightly rough. Subsequently, spinous papules appeared at the sites of the hair follicles, first involving the anterolateral aspect of the thighs and the posterolateral aspect of the upper part of the forearms. The eruption gradually spread to the extensor surface of both upper and lower extremities, the shoulders and the lower part of the abdomen, and to a less extent to the chest, back and buttocks. In some cases the skin was darker than normal, turning a dull slate color. There was absence of visible sweating, and the articular folds, which are usually moist, were dry and covered with closely adherent, delicate scales. The normal markings on the surface of the skin were exaggerated in places, giving it a finely wrinkled appearance.
>
> The follicular papules varied in size according to the stage of development and the degree of perifollicular infiltration. The largest were approximately 5 mm. in diameter, hemispherical, rather firm and usually deeply pigmented. The hyperpigmentation extended in a narrow zone beyond the base of the lesion. Each papule held in its apex a keratotic plug which in most instances projected above the surface of the lesion as a hard spinous process, or was covered by a loosely adherent scale that bridged the occluded follicular recess. When expressed, the plugs left gaping central craters in the summits of the papules. The eruption was usually abundant and sym-

[33] C. N. Frazier and C. K. Hu, *Arch. Internal Med.* **48**, 507 (1931).

[34] C. N. Frazier and C. K. Hu, *Arch. Dermatol. and Syphilol.* **33**, 825 (1936).

metrical, but occasionally the lesions were few and widely scattered, or more rarely, restricted to a single area.

An "acne-like" eruption on the face, differing from acne vulgaris with respect to dryness of the skin and rarity of pustulation, was also described. Microscopic study of the follicular lesions indicated that "the primary process was an extensive epithelial keratinization, resulting in mechanical occlusion of the hair follicles and sweat ducts, and in secondary degeneration of the sebaceous and sweat glands." With improved diet and cod liver oil therapy (up to 30 ml. daily), the skin lesions improved, but more slowly than the ocular symptoms; even after 2 months the skin had not regained its normal texture and appearance.

In a later report Frazier and Hu[34] review and extend their previous findings, commenting on cutaneous lesions observed in 180 patients with, and 27 patients without, evidence of xerophthalmia. Of 91 patients, 1 month to 15 years of age, about equally divided as to sex, only 6 showed follicular hyperkeratosis and 26 simple xerosis of the skin. In 103 patients 16 to 30 years of age, almost all males, 47 showed follicular lesions and 12 xeroderma. They emphasized that the papular eruption was not seen in infants and rarely occurred before puberty, and that simple xerosis of the skin, which frequently preceded the onset of follicular lesions in adults, may represent a common manifestation of vitamin A deficiency in infants and children.

It is of interest to note that follicular hyperkeratosis, responding to cod liver oil therapy, was described as frequently appearing before the onset of keratomalacia,[33] and often before, or sometimes in the absence of, signs of xerophthalmia.[34] Reiss,[35] reporting from Shanghai, described similar follicular lesions of at least 8 months' duration in 7 rug-factory workers showing only night blindness; only 4 showed complete recovery with cod liver oil therapy. With increased tendency to accept follicular hyperkeratosis as pathognomonic of avitaminosis A in the absence of other well-established evidence of the deficiency state, it is natural to question whether investigators in various countries (through laxity in diagnostic criteria and lack of facilities for adequate evaluation of the vitamin A status in patients) may have unwittingly included among their cases instances of follicular eruptions unrelated to lack of vitamin A, especially when dealing with children.

b. Studies in Other Countries

The findings of Frazier and Hu[33] were independently confirmed by the studies of Nicholls[36] on laborers in Africa and convicts in Ceylon, and those

[35] F. Reiss, *Chinese Med. J.* **50**, 945 (1936).

[36] L. Nicholls, *Indian Med. Gaz.* **68**, 681 (1933).

of Loewenthal[37] on prisoners in East Africa. Nicholls proposed the term "phrynoderma," meaning like toad skin, for the papular eruption. Although this term has been used chiefly by investigators in Africa and India, the term "follicular hyperkeratosis" proposed by Frazier and Hu[34] is perhaps more descriptive of the histopathologic lesion and more commonly used. In the two studies mentioned above there was again the combination of high incidence of ocular symptoms, diets very deficient in vitamin A, dry and slate-grey skin, and follicular lesions which responded to cod liver oil therapy (usually 1 oz. daily) more slowly than did the ocular symptoms.

These early investigators were fully aware of the similarity between the lesions ascribed to lack of vitamin A and those of other skin diseases involving the pilosebaceous follicles. In addition to the therapeutic response to cod liver oil, distribution of the lesions and infrequency of pustulation seem to rule out acne vulgaris; the rarity of the papular lesions in postpubertal females, and in adolescent children, seemed incompatible with keratosis pilaris; the incidence, and the lack of evidence of familial tendency or of dyskeratotic changes histologically, seemed to exclude the relatively rare condition of Darier's disease. As further evidence concerning the specificity of the lesions, there was the demonstrated effectiveness of halibut liver oil by Loewenthal in 2 patients, and by Frazier and Hu[34] in 1 patient, and of carotene injected intramuscularly in 1 patient by Frazier and Li.[38]

Further confirmation, based upon the macroscopic and microscopic character of the cutaneous lesions in adult subjects, and their response to vitamin A therapy, is represented largely by the reports of Giblin[39] from New Guinea (1 case), Radhakrishna Rao[40, 41] from Southern India, Fasal[22] from the Malay states, and Steffens *et al.*[42] (1 case) and Youmans and Corlette[43] from this country. The recent report of Nichol,[44] based on a nutritional survey in Nigeria in which careful attention was given to the macroscopic features of the cutaneous lesions and their relation to other symptoms of avitaminosis A, also provides strong evidence regarding the specificity of the lesions. In these confirmatory reports, varying degrees of ocular manifestations of vitamin A deficiency were common in the subjects examined,

[37] L. J. A. Loewenthal, *E. African Med. J.* **10**, 58 (1933); *Arch. Dermatol. and Syphilol.* **28**, 700 (1933).

[38] C. N. Frazier and H. C. Li, *China Med. J.* **54**, 301 (1938).

[39] W. E. Giblin, *Med. J. Australia* **1**, 202 (1936).

[40] M. V. Radhakrishna Rao, *Indian J. Med. Research* **24**, 727 (1937).

[41] M. V. Radhakrishna Rao, *Indian J. Med. Research* **25**, 39 (1937).

[42] L. F. Steffens, H. L. Bair, and C. Sheard, *Am. J. Ophthalmol.* **23**, 1325 (1940).

[43] J. B. Youmans and M. B. Corlette, *Am. J. Med. Sci.* **195**, 644 (1938).

[44] B. M. Nichol, *Brit. J. Nutrition* **3**, 25 (1949).

except for the human volunteer studied by Steffens *et al.* and the patients studied by Youmans and Corlette; in these subjects impairment of dark adaptation was absent or questionable, but there was effective response of the lesions to highly potent concentrates of vitamin A. One report by Radhakrishna Rao[41] is notable in that it describes cases of advanced keratomalacia with skin changes comparable to those observed by Pillat[16] and presents for the first time the histopathologic changes involved.

Although the Sheffield human experiment[1] was unfortunately inconclusive concerning the specificity of skin lesions of vitamin A deficiency, it indicates that 2 years or more are required to seriously deplete the vitamin A stores of healthy young adults. During the second year of deficiency, plasma levels of vitamin A were reduced to a critical point (less than 40 I.U. per 100 ml.) in only 4 subjects; only 3 of these had evidence of night blindness, and only 1 showed cutaneous changes which suggested inadequate vitamin A. These improved slowly over a period of 6 months of therapy (1300 I.U. daily—as compared to 80,000 I.U. daily, used in the studies of Steffens *et al.*[42]). Had it been possible to extend these depletion periods into the third year, it seems likely that more convincing cutaneous manifestations would have appeared.

The results of another human experiment, the "Minnesota experiment"[45] on the effects of prolonged partial starvation, suggest that general undernutrition may play some part in the genesis of cutaneous lesions attributed to avitaminosis A. In 24 of 31 subjects maintained for 23 weeks on a partial starvation diet (a low-fat diet providing 1810 I.U. of vitamin A daily, chiefly as carotene; this represents a submarginal daily intake of vitamin A), there was noted a mild to moderate papular eruption which bore certain resemblances, grossly and histologically, to the follicular hyperkeratosis of vitamin A deficiency. Plasma levels of vitamin A were as high at the end of the experimental period as at the beginning. A dry scaly skin and dry lusterless hair were also noted in more than half the subjects. This report also cites numerous references to descriptions of similar alterations of the skin during famines, wars, and sieges. On the other hand, those who studied the nutritional status of civilian population, internees, and refugees in Europe immediately after World War II found surprisingly little evidence of skin lesions or other clinical evidence of avitaminosis A. They attributed this to the fact that when the normal food supply is restricted the consumption of greens, vegetables, and cereal grains increases markedly and provides a reasonably abundant, and sometimes an excess, supply of carotene.

[45] A. Keys, J. Brozek, A. Henschel, O. Mickelsen, and H. L. Taylor, The Biology of Human Starvation, Vols. 1 and 2. University of Minnesota Press, Minneapolis, 1950.

2. Macroscopic Features in Infants and Children

The early reports dealing with xerophthalmia in infants and children make only occasional reference to skin changes, and these relate chiefly to dryness, wrinkling, and generalized xerosis[9-11] which to some investigators[30, 32] appear to reflect dehydration and emaciation so often apparent. On the other hand, following the characterization of cutaneous lesions in adults there have appeared numerous reports dealing with follicular hyperkeratosis in children which are often cited as confirming the former studies. It seems that inaccurate diagnosis, inadequacy of ancillary data, and unjustified interpretation of data in some of these studies are largely responsible for confusion and misgivings concerning the specificity of follicular lesions as a manifestation of avitaminosis A. If one considers the evidence presented separately, and apart from that pertaining to adult man, it seems unnecessary to take the viewpoint of Stannus,[46] who, after a critical appraisal of the literature, concluded that the cutaneous alterations ascribed to avitaminosis A are none other than the rather common dermatologic disorder, keratosis pilaris; however, he admits that inadequate vitamin A may be an important factor in the genesis of keratosis pilaris under certain conditions.

Reference has been made to a report of Frazier and Hu[34] stating that follicular hyperkeratosis rarely occurred before the age of puberty, and that the usual cutaneous manifestation of A deficiency in children was xeroderma. A continuation of these studies,[47] based upon routine examination of patients admitted to the pediatric service of Peiping Union Medical College over a 5-year period, brought forth evidence that typical lesions of follicular hyperkeratosis do occur before puberty, but that the degree of involvement of the pilosebaceous follicle is quite limited in infants and becomes more marked with increasing age. These conclusions are supported by 5 case histories, with histologic studies of skin biopsies, illustrating the maximal changes observed at successive age periods (69 days, 16 months, 2 years 10 months, 5 years, and 15 years of age), and recapitulating the progressive phases of development of skin lesions seen in young adults. Delay in functional maturity of pilosebaceous follicles and variations in hormonal influences on the skin during sexual development were regarded as factors modifying the effect of vitamin A deficiency upon the integument. With reference to keratosis pilaris they state: "We have never included instances of this minor, but widely prevalent, lesion among the cases of vitamin A deficiency that we have reported."

In contrast to the rarity of follicular eruptions in children in the experience of Frazier and Hu, observers in other countries have reported a rela-

[46] H. S. Stannus, *Proc. Roy. Soc. Med.* **38**, 237 (1945).

[47] C. N. Frazier, C. K. Hu, and F. Chu, *Arch. Dermatol. and Syphilol.* **48**, 1 (1943).

tively high incidence of the lesions in children of school age, often in association with a low incidence of ocular symptoms referable to vitamin A deficiency. Nicholls[48] observed typical skin lesions in about one-fourth of 4380 children in Ceylon, and Loewenthal[49] in about one-third of 952 children examined in Uganda, of which only 40 had ocular symptoms. The general picture of a mild "goose-flesh" type of eruption in very young children and small papules in older children, progressing to the more conspicuous follicular lesions in adults conforms to that observed in China. Aykroyd and associates[50, 51] observed a lower incidence in children in South India and, largely on the basis of poor correlation between estimated dietary intake and frequency of skin and ocular manifestations, felt that lack of vitamin A was only one factor, and perhaps not the major one, in the genesis of the follicular lesions. Yet, the histopathology of the lesions in children and adults from the same local area, as described by Radhakrishna Rao,[40, 41] indicates a true avitaminosis A.

Fasal[22] reports finding follicular lesions in 2% of 1482 Malay, and 25.6% of 657 Tamil, children. Although the incidence of Bitot's spots did not parallel that of the skin lesions in the two groups, striking differences in the dietary intake of vitamin A and the response of the lesions to red palm oil and vitamin A concentrates strongly implicated lack of vitamin A. Fasal states that the lesions closely resemble those of ichthyosis follicularis and comments on their asymmetrical intensity in adults due to local pressure and friction, such as resting of forearm on the thigh in weeding operations and carrying of infants on the hip.

More recently, Ramalingaswami[52] has reported a high incidence of Bitot's spots, keratomalacia, severe diarrhea, dry inelastic skin, and follicular hyperkeratosis in children attending the nutrition clinic at Coonoor, South India. The estimated dietary intake of vitamin A was 460 I.U., and plasma vitamin A levels determined on 6 children with severe diarrhea ranged from 0 to 32 I.U. per 100 ml. Administration of a vitamin A concentrate (72,000 I.U. of vitamin A daily) gave dramatic control of the diarrhea in about 48 hours and a more gradual improvement in the other manifestations of the deficiency state. On the other hand, Pal[29] reports on 70 cases of avitaminosis A, mostly children 3 to 8 years of age, attending a medical school Out-Patient Department in Calcutta; the majority showed ocular manifestations ranging from night blindness to keratomalacia, yet only 4 cases of follicular hyperkeratosis were recognized.

[48] L. Nicholls, *Indian Med. Gaz.* **69**, 241 (1934); **70**, 14 (1935); **70**, 550 (1935).
[49] L. J. A. Loewenthal, *Ann. Trop. Med. Parasitol.* **29**, 349, 407 (1935).
[50] W. R. Aykroyd and K. Rajagopal, *Indian J. Med. Research* **24**, 419, (1936).
[51] W. R. Aykroyd and B. G. Krishnan, *Indian J. Med. Research* **24**, 707 (1937).
[52] V. Ramalingaswami, *Indian J. Med. Sci.* **2**, 665 (1948), cited in *Nutrition Revs.* **3**, 38 (1949).

In studies such as those of Pemberton[53] in England, describing a mild follicular hyperkeratosis in 5 % of 3000 school children with no other indication of low vitamin A, the specificity of the skin lesions is questionable. A report from the United States[54] deals with 9 children showing mild papular lesions associated only with subnormal biophotometer readings. The sex distribution, the familial tendency, amelioration during warm weather, and the fact that 100,000 to 300,000 I.U. of vitamin A daily over a period of 2 to 4 months effected only "nearly complete recovery" leaves some doubt as to the specificity of the lesions described. The question of high levels of vitamin A therapy (75,000 I.U. or more, daily) and the effect upon dermatologic disorders presumably unrelated to avitaminosis A is discussed in a later section (p. 159).

A recent report by Marmelzat[54a] from Galveston, Texas, describes a rare combination of Bitot's spots, typical follicular hyperkeratosis lesions, and metabolic calcinoses of both kidneys observed in a 10-year-old white girl. The skin over the abdomen, thorax, posterior and lateral surfaces of the arm, elbows, and knees presented minute horny papules about 2 mm. in diameter, giving a "grater-like" feel to the surface. Biopsy revealed moderate hyperkeratosis and keratotic plugging of sebaceous follicles. There was regular nocturnal fever (100 to 102° F.) during a 4-week period of observation on a high-caloric diet supplemented by iron and vitamin B supplements; fever and conjunctival and skin lesions remained the same. With 25,000 I.U. of vitamin A orally, the Bitot's spots disappea ed in less than 24 days; after a little over 2 months, when the patient was lost from observation, the follicular lesions had disappeared except for a few on the arms. Although this was regarded as a conditioned deficiency, secondary perhaps to giardiasis, the observations and findings closely parallel those reported from Oriental countries and further confirm the generally accepted relationship between avitaminosis A and follicular hyperkeratosis in children.

The frequent lack of correlation between incidence of skin lesions and of other evidence of vitamin A deficiency in studies on children is probably due as much to differences in and inadequacy of criteria of vitamin A deficiency employed by the various observers as to variability within the groups of children studied. No correlations have been made between age of children and incidence of lesions. One must also consider the possibility that, depending on the role of vitamin A depletion or the influences of metabolic (diarrhea, liver dysfunction) or environmental (exposure of body areas, clothing, hygiene) factors, cutaneous and ocular manifestations of

[53] J. Pemberton, *Lancet* **I**, 871 (1940).

[54] E. Lehman and H. G. Rapaport, *J. Am. Med. Assoc.* **114**, 386 (1940).

[54a] W. L. Marmelzat, *Arch. Dermatol. and Syphilol.* **63**, 759 (1951).

avitaminosis A may occur separately as well as concurrently either in children or adults. Their acceptance as typical, when isolated, phenomena should be based upon their disappearance within 1 to 2 months under moderate dosage of vitamin A concentrate and, if possible, on supporting evidence of low plasma vitamin A levels or low dietary intake over prolonged periods. Where subjects exhibit angular stomatitis as well, vitamin B-complex therapy is often prerequisite to an adequate response to vitamin A therapy.[22, 50]

Conclusions based upon those studies where the greatest care has been exercised in dermatologic diagnosis, biopsy examination, and correlation with other evidence of avitaminosis A indicate that infants and children up to 4 to 5 years of age rarely show more than generalized xerosis of the epidermis, and that during later childhood and adolescence there may be superimposed upon this a mild type of follicular hyperkeratosis indistinguishable from, but less pronounced than, that characterizing avitaminosis A in adults.

3. Histopathology

The cutaneous lesions of avitaminosis A represent a primary hyperkeratinization and hyperplasia of the epidermis, including the lining of the hair follicles and sebaceous glands. In a strict sense this is not a true metaplasia, as is seen in other epithelial surfaces affected by lack of vitamin A, but rather an accentuation of a process of progressive keratinization normally inherent in this epithelial layer. Decreased function of sweat glands and the follicular eruption which is so prominent a gross feature are the later results of this primary process.

In infants and young children under 5 years of age, before the pilosebaceous follicle has fully matured, there is usually only a simple xerosis, or xeroderma. However, there may be moderate hyperkeratinization of the follicle lining. The superficial epidermis is sometimes reduced in thickness[47] rather than hyperplastic. The stratum corneum is usually several times its normal thickness and may cause blockage of sweat ducts.

A more exaggerated picture is seen in adults with advanced keratomalacia, as described first by Pillat[16] and studied microscopically by Radhakrishna Rao.[41] The stratum corneum forms a broad network, or even horny plates, and gives rise to a rather abundant desquamation of fine scales. The stratum lucidum and stratum granulosum show no appreciable change. Melanin increases in the basal layers, and the epidermis may be thinned rather than hypertrophic. This atrophic thinning, often associated with flattening of the papillary layer of the dermis, may reflect general undernutrition. Sebaceous glands are greatly reduced in number; sweat ducts are occluded by keratinous material, but the coiled glands are usually

normal in appearance though probably hypofunctional. The lining of hair follicles is usually hyperkeratotic, and the follicles are sometimes moderately dilated with horny plugs enveloping coiled remnants of the hair, but the plugs rarely project above the surface of the skin. Except for occasional mild perifollicular infiltration, the corium is normal. There is no adequate explanation as to why the follicular reactions are so minimal despite the excessive hyperkeratosis in these instances of advanced avitaminosis A, while the intensity of the two reactions is reversed in the more common type of cutaneous change, referred to as follicular hyperkeratosis, usually associated with less marked ocular symptoms or at times with absence of such symptoms.

In typical follicular hyperkeratosis the follicle cavity becomes greatly distended by a conical or hemispherical plug consisting of concentric layers of keratinized cells cemented together by sebum, within which coiled remnants of the hair may be seen. The papules vary in size but rarely exceed 5 mm. in diameter. The dilated follicle may be bridged by a loosely adherent scale, or the plug may project above the surface of the skin. When the plugs are expressed, or when they are shed spontaneously after a week or two of vitamin A therapy, gaping holes are left. These gradually diminish in size and rarely leave scars.

The epidermis between follicles shows varying degrees of hyperplasia and hyperkeratosis; this is most marked as one approaches the margin of the follicle where increased pigment in cells of the basal layers is also a prominent feature, accounting for the hyperpigmentation often grossly visible at the base of the involved follicles. Despite the abundance of keratinized cells on the epidermis and in follicle plugs, providing a culture medium for many bacteria, skin infections are no more common than in normal individuals. Various observers have commented on the rarity of pustulation, or on the fact that acne eruptions on the face, or intermingled with the follicular lesions in other areas of the body, show a much lesser degree of pustulation in individuals exhibiting follicular hyperkeratosis. The dermis is normal except for cellular infiltration or mild inflammatory reactions occasionally seen in the perifollicular area.

The short ducts of the sebaceous glands, lined by stratified squamous epithelium continuous with that of the follicle cavity, also exhibit hyperkeratinization which may lead to blockage of the ducts. There is extensive atrophy of sebaceous glands related to hyperkeratotic follicles, but no evidence of hyperkeratosis of the glandular epithelium. Absence of cystic dilation of the glands suggests that duct blockage is not an important factor. None of the observers have tried to explain this atrophy. It seems possible that the cause may lie in the altered duct epithelium, which is generally regarded as the source of new cells for the epithelium of the glands proper.

The latter epithelium is stratified in type, but its more superficial cells are constantly undergoing a fatty metamorphosis to produce, by the process of holocrine secretion, the waxy sebum. Lack of vitamin A might merely inhibit the mechanism of secretory metamorphosis, but it seems more plausible that failure of the altered duct epithelium to replace cells lost in the secretory process is the primary cause of sebaceous gland atrophy. Observers have generally ignored the arrectores pilorum muscles of the pilosebaceous apparatus, so that it is only presumed that they are normal.

The sweat glands, which are also derivatives of the epidermis, possess narrow, coiled excretory ducts continuous with excretory canals in the epidermis which are merely intracellular clefts lined by epidermal cells. Hyperkeratinization of the epidermis in avitaminosis A readily leads to blockage of these excretory canals. The cuboidal epithelium of the duct exhibits keratinizing metaplasia, especially in its terminal portion, contributing also to blockage. Some dilation of acini occurs, probably as a result of duct obstruction, but no metaplasia of glandular epithelium has been observed. This is in accord with the promptness with which moistness of the skin reappears after vitamin A therapy.

F. MASSIVE VITAMIN A THERAPY IN CLINICAL MEDICINE

Certain similarities between follicular hyperkeratosis and other skin disorders involving pilosebaceous follicles and sweat glands, and postulations that some of these lesions might represent a local, and possibly hereditary, metabolic defect in utilization of vitamin A, have led dermatologists to explore the therapeutic value of massive doses of vitamin A. The dosage used has usually varied from 75,000 to 400,000 I.U. of vitamin A daily, in the form of potent concentrates; this represents 15 to 80 times the recommended daily allowance or 60 to 320 times the minimal daily requirement, for adult man. This range of intake, extending over 3 to 6 months, produces symptoms of hypervitaminosis A in young children, as discussed below. Although these studies are in an exploratory stage, reference to certain of the observations reported seems pertinent, since they have particular bearing upon the preceding discussion on cutaneous lesions and upon the question of hypervitaminosis A to be dealt with later.

Particular attention has been given to keratosis follicularis (Darier's disease), a relatively rare condition, characterized by a benign dyskeratosis associated with a moderate hyperkeratosis which leads to formation of keratotic plugs not only in hair follicles but elsewhere on cutaneous surfaces.[55] The papular lesions are at first pale and discrete and later become brown or reddish as they tend to coalesce into papillomatous masses; the palms and soles may become horny and thickened.

[55] F. A. Ellis, *Arch. Dermatol. and Syphilol.* **50**, 27 (1944).

In 1941, Peck *et al.*[56] reported the beneficial response of 4 patients with Darier's disease to high vitamin A therapy. This was confirmed by others prior to their later summary of experience with 10 patients,[57] and by subsequent reports.[58-61] Although the response of patients has varied widely and has been negative in some instances, the good responses do not seem explicable on the basis of spontaneous remission. When considered as a group, the majority of patients have shown low plasma levels of vitamin A, associated with low carotene levels in some studies[60] but not in others,[57] and with dark dysadaptation in some[57] but not in others.[58] After vitamin A therapy, blood levels have been restored to normal or somewhat above normal,[57] or have sometimes reached exceedingly high levels.[60] The majority of patients have shown striking improvement in the skin lesions, usually after several months of therapy. With discontinuation of therapy, a tendency for lesions to recur and for vitamin A blood levels to decrease below normal has been noted.[57] The findings referred to have much in common with a hereditary hyperkeratosis in mice which is considerably modified by high vitamin A therapy.[62] It is postulated that a great excess of vitamin A may overcome a metabolic defect in the ability of the liver or cutaneous structures to utilize vitamin A,[57] or may produce a state in which the rate of absorption exceeds liver storage to the point where vitamin A reached tissues from which it is excluded by disease.[60]

In pityriasis rubra pilaris (Devergie's disease), a rare and hereditary disease of which a major characteristic is hyperkeratosis of hair follicles, vitamin A blood levels are said to be within normal limits;[63, 64] yet 100,000 to 200,000 I.U. of vitamin A daily causes pronounced involution of the lesions.[63] Somewhat the same observations have been made in other hyperkeratotic states such as keratosis palmaris et plantaris, ichthyosis and pachyonychia,[65, 66] and local callosities.[67] In a variety of skin diseases not

[56] S. M. Peck, L. Chargin, and H. Sobotka, *Arch. Dermatol. and Syphilol.* **43**, 223 (1941).

[57] S. M. Peck, A. W. Glick, H. Sobotka, and L. Chargin, *Arch. Dermatol. and Syphilol.* **48**, 17 (1943).

[58] A. Carleton and D. Steven, *Arch. Dermatol. and Syphilol.* **48**, 143 (1943).

[59] Z. A. Leitner and T. Moore, *Brit. J. Dermatol. Syphilis* **60**, 41 (1948).

[60] Z. A. Leitner, *Brit. J. Nutrition* **5**, 130 (1951).

[60a] Z. A. Leitner and T. Moore, *Lancet* **I**, 262 (1946).

[61] A. Rostenberg and W. M. Siskind, *Arch. Dermatol. and Syphilol.* **61**, 135 (1950).

[62] F. C. Fraser, *Can. J. Research* **27**, 179 (1949).

[63] A. L. Weiner and A. A. Levin, *Arch. Dermatol. and Syphilol.* **48**, 288 (1943).

[64] Z. A. Leitner, *Brit. J. Dermatol. Syphilis* **59**, 407 (1947).

[65] A. D. Porter and H. Haber, *Brit. J. Dermatol. Syphilis* **62**, 355 (1950).

[66] A. D. Porter, *Brit. J. Dermatol. Syphilis* **63**, 123 (1951).

[67] J. V. Straumfjord, *Northwest Med.* **41**, 7 (1942).

associated with hyperkeratosis, vitamin A blood levels are normal.[60,68] Intensive vitamin A treatment has been reported as beneficial in mal de Meleda,[69] postmenopausal vagina cornification,[70] chalazion,[71] and in progressive deafness and tinnitus.[72-74] Its reported value in the treatment of acne has not been supported by recent studies.[75, 76] There is also an interesting but unconfirmed observation that vernix caseosa is a manifestation of vitamin A deficiency, or at least can be reduced by administration of vitamin A to the pregnant mother.[77] Many of the reports mentioned above are cited largely to emphasize trends of interest and application of knowledge of vitamin A. Only time and acquisition of many more data can determine the validity of the observations and conclusions so far recorded.

G. HYPERVITAMINOSIS A

For twenty years or more it has been recognized that animals given a great excess of vitamin A, usually more than 10,000 times their minimal requirement, show evidence of toxic effects. These include anorexia, diarrhea, disheveled fur, decalcification and spontaneous fractures of bones, scurvy-like hemorrhages, and a variety of lesions in visceral organs. The effects are not related to an excess of vitamin D, or fatty acids or toxic substances present in fish liver oils often used in such studies, for they can be produced by highly potent concentrates of vitamin A or by synthetic vitamin A acetate; moreover, fish liver oils in which vitamin A has been inactivated do not produce these symptoms. Perhaps the most characteristic tissue injury, histologically and roentgenographically, is an acceleration of bone remodeling during skeletal maturation to the point where resorption of bone is greater than deposition of new bone; this results in the formation of defective bone which is readily subject to spontaneous fracture.

It has also been recognized for many years that polar bear liver, an unusually potent source of vitamin A, is shunned by Eskimos and polar explorers because of its toxicity, even when used as a food for Eskimo dogs.[78] It is only during the past seven years, however, that a state of

[68] T. Cornbleet, H. Popper, and F. Steigmann, *Arch. Dermatol. and Syphilol.* **49**, 103 (1944).

[69] M. J. Brunner and D. L. Fuhrman, *Arch. Dermatol. and Syphilol.* **61**, 820 (1950).

[70] L. A. Platt, *Am. J. Clin. Pathol.* **21**, 38 (1951).

[71] C. S. Hickey, *Eye, Ear, Nose Throat Monthly* **30**, 488 (1951).

[72] M. J. Lobel, *Eye, Ear, Nose Throat Monthly* **28**, 213 (1949).

[73] J. R. Anderson, H. J. Zoller, and L. W. Alexander, *Eye, Ear, Nose Throat Monthly* **29**, 75 (1950).

[74] H. W. Bau and L. Savitt, *Eye, Ear, Nose Throat Monthly* **30**, 83 (1951).

[75] F. W. Lynch and C. D. Cook, *Arch. Dermatol. and Syphilol.* **55**, 355 (1947).

[76] G. A. Mitchell and T. Butterworth, *Arch. Dermatol. and Syphilol.* **64**, 428 (1951).

[77] J. V. Straumfjord, *Western J. Surg. Obstet. Gynecol.* **48**, 341 (1940).

[78] K. Rodahl and T. Moore, *Biochem. J.* **37**, 166 (1943).

toxicity due to an excess intake of vitamin A has been recognized in young children. In view of the increasing usage of oleum percomorphum, halibut liver oil, and other highly potent preparations of vitamin A, it is not surprising that overzealous or careless parents and grandparents have been responsible for the appearance of a new clinical syndrome. The causal factors vary—careless measurement of concentrates, failure to distinguish between potent concentrates and cod liver oil or between drops and teaspoons as measures of dosage, and the idea that if a small amount of a concentrate is good for the child a larger amount should be better.

Since 1944, when Josephs[79] first recognized the syndrome of hypervitaminosis A in man, ten reports have appeared recording 19 cases, all in the United States. Seven of these were reported in 1950 by Caffey,[80] who presents an excellent description of the symptomatology and roentgenographic features and reviews 8 cases previously reported in the literature. Since then 2 cases have been reported by Gribetz *et al.*,[81] (who present a tabular summary of 14 cases previously reported) and 1 case each by Berrey[82] and Bair.[83] Probably many other instances have occurred in which symptoms were not severe enough to be called to the attention of a physician, or in which a correct diagnosis was not made.

The age of the 19 patients ranged from 12 to 37 months. The estimated vitamin A intake varied from 75,000 to 500,000 I.U. per day, generally over a period of 3 to 6 months. The chief symptoms were: anorexia, hyperirritability, painful but minimal soft tissue swellings over areas of skeletal exostoses of long bones, limited motion of extremities, cortical thickening of long bones, sparsity of scalp hair, pruritic rash, and hepatomegaly without splenomegaly. Vitamin A plasma levels were exceptionally high and a valuable diagnostic feature, equaled only by the dramatic and rapid disappearance of symptoms within a week or so following discontinuation of vitamin A supplements. Since recovery has always been complete, nothing is known regarding histopathologic changes in bone and visceral organs. Discontinuation of the vitamin resulted in recovery, usually in a week or so, in all cases. There is as yet no direct evidence that hypervitaminosis A induces in the bone and other tissues of infants changes which are comparable to those observed in experimental animals. It seems reasonable to assume such would be the case if the hypervitaminotic A state persisted for a sufficient period of time.

In man, prolonged and excessive intake of vegetables high in carotene,

[79] H. W. Josephs, *Am. J. Diseases Children* **67**, 33 (1944).

[80] J. Caffey, *Am. J. Roentgenol. Radium Therapy* **65**, 12 (1951); *Pediatrics* **5**, 672 (1950).

[81] D. Gribetz, S. H. Silverman, and A. E. Sobel, *Pediatrics* **7**, 372 (1951).

[82] B. H. Berrey, *Pediatrics* **6**, 78 (1950).

[83] G. Bair, *J. Am. Med. Assoc.* **146**, 1573 (1951).

and certain metabolic disorders which interfere with the conversion or other utilization of carotene, leads to an intense deep yellow coloration of the skin and subcutaneous tissues known as xanthosis cutis, which is often difficult to distinguish from jaundice, but produces no acute symptoms or damaging effects on tissues.

IX. Requirements

A. OF ANIMALS

FRED H. MATTSON

1. General Considerations

Investigations as to the vitamin A requirements of animals have been limited mainly to laboratory animals and species having an economic importance. With the exception of the rat, the requirements of even the routinely used species of laboratory animals have not been established with certainty. However, from the more thoroughly investigated species, a common pattern relating the vitamin A requirement of mammals to body size has become apparent. This concept was proposed in 1935 by Guilbert and Hart.[1] Studies carried out subsequently by these and other workers have further strengthened this concept. It appears that, for mammals, 24 γ of β-carotene (equivalent to 40 I.U. of vitamin A) or 20 I.U. of vitamin A per kilogram of body weight is the minimum daily requirement to support growth and prevent gross symptoms of deficiency. For storage, reproduction, and lactation considerably higher requirements must be met. The requirements of chickens and turkeys per unit of body weight are higher than those of mammals.

It will be noted that a double standard has been arrived at, depending on whether vitamin A or carotene is fed. Under the conditions of the biological assay, 3 γ of vitamin A alcohol is equivalent to 6 γ of β-carotene. This relationship, however, holds only under those specified conditions. When the intake is much in excess of that used under assay conditions, the relative potency of the carotene drops off with increasing levels of intake in both mammals[2] and birds.[3]

Under practical feeding conditions, a mixture of carotenoids, some of which are precursors of vitamin A, as well as vitamin A itself, are used. In using such a feed, account must be taken of the biological potency of the

[1] H. R. Guilbert and G. H. Hart, *J. Nutrition* **10**, 409 (1935).
[2] H. R. Guilbert, C. E. Howell, and G. H. Hart, *J. Nutrition* **19**, 91 (1940).
[3] R. B. Nestler, J. V. Derby, and J. B. De Witt, *J. Nutrition* **36**, 323 (1948).

individual carotenoids. Moreover, within recent years, particularly in the laboratories of Deuel and Zechmeister,[4] experiments have shown that the stereoisomeric form of a particular carotenoid will markedly influence its biological potency. For example, it was found that neo-β-carotene U had only 38% of the potency of all trans-β-carotene. These isomeric changes can be of considerable importance, depending on the treatment to which the carotenoid-containing feeds have been subjected. If corrections are not made for the presence of isomers, estimates as much as 30% in excess of the true potency can result.[5]

Many of the studies on the vitamin A requirements of animals were made before these differences in biopotency were fully appreciated. This would account for some of the variations among the reported results leading to estimates which are in error on the high side. Since under practical feeding conditions, mixtures of vitamin A and carotenoids occurring in natural foods are fed, these values have the advantage of more general applicability.

In 1943 the National Research Council's Committee on Animal Nutrition established a series of subcommittees to prepare nutritional standards for domestic animals of economic importance. These subcommittees were composed of members who had made a speciality of the nutrition of a particular species. The recommended allowances are revised when the availability of new data warrants. These values are to be recommended for practical feeding conditions, since they were established for this purpose and contain, moreover, an adequate safety factor to meet exigencies that might arise.

It is not possible to summarize the requirements of all animals on a uniform basis, such as diet composition or body size, because of variations in experimental technique used by various investigators. Where sufficient data were given in the original publication, the values have been recalculated to allow the use of a single basis for any one species. For the sake of uniformity and to allow comparisons between species, all values are reported in terms of international units of vitamin A. In the case of cattle, horses, swine, and sheep most of the values were obtained by calculation from their carotene equivalent, since carotene is usually administered as the source of the vitamin in the studies on these species.

2. Rats and Mice

Numerous criteria have been used in studying the vitamin A requirement of the rat. The final recommended value, of course, depended on the standard employed. The results of some of these studies are summarized in Table XVIII. From these data, it would seem that the daily intake allow-

[4] L. Zechmeister, *Vitamins and Hormones* **7**, 57 (1949).

[5] E. M. Bickoff and C. R. Thompson, *J. Assoc. Offic. Agr. Chemists* **32**, 775 (1949).

ing optimal growth is approximately 100 I.U. per kilogram of body weight when vitamin A is fed. When carotene is to serve as the source of the vitamin, a considerably larger amount is required. Irving and Richards[11] have reported data which they interpret as meaning an increase in vitamin A requirement with age. The results obtained by Little *et al.*,[10] where liver storage was measured following the feeding of graded doses of vitamin A, would seem to support this hypothesis. However, it is possible that the results obtained merely represented a greater requirement as the body size

TABLE XVIII

ESTIMATES OF THE VITAMIN A REQUIREMENT OF THE RAT

Level fed	Criterion used	References
I.U. per kilogram of body weight per day		
18–22	Vaginal smear	6, 2
20	Vaginal smear	7
20	Gross and histological evidence of deficiency	8
50–80	Liver storage	7
80	Growth	7
100	Growth and longevity	9
135	Liver storage	6, 2
I.U. per rat per day		
12	Liver storage	10
20	Blood level	11
25	Growth	8
50	Blood level	8
100	Liver storage	8
I.U. per gram of food		
12	Growth and longevity	12
12	Growth and breeding	13

of the animals increased. Of considerable interest are the results obtained by Sherman[15] where growth, longevity, and reproductive period were

[6] H. Goss and H. R. Guilbert, *J. Nutrition* **18**, 169 (1939).
[7] E. C. Callison and V. H. Knowles, *Am. J. Physiol.* **143**, 444 (1945).
[8] J. M. Lewis, O. Bodansky, K. G. Falk, and G. McGuire, *J. Nutrition* **23**, 351 (1942).
[9] H. E. Paul and M. F. Paul, *J. Nutrition* **31**, 67 (1946).
[10] R. W. Little, A. W. Thomas, and H. C. Sherman, *J. Biol. Chem.* **148**, 441 (1943).
[11] P. B. Horton, W. A. Murrill, and A. C. Curtis, *J. Clin. Invest.* **20**, 387 (1941).
[12] H. C. Sherman and H. L. Campbell, *Proc. Natl. Acad. Sci. U. S.* **31**, 164 (1945).
[13] H. C. Sherman and H. Y. Trupp, *J. Nutrition* **37**, 467 (1949).
[14] J. T. Irving and N. B. Richards, *Nature* **144**, 908 (1939).
[15] H. C. Sherman, H. L. Campbell, M. Udiljak, and H. Yarmolinsky, *Proc. Natl. Acad. Sci. U. S.* **31**, 107 (1945).

studied. Although normal growth was obtained when the animals were fed vitamin A at a level of 100 I.U. per kilogram of body weight per day, survival time and the reproductive period were extended when the level of intake was increased to 200 I.U. and still more when 400 I.U. were fed. From these results, it would appear that the optimal level of vitamin A intake may be far in excess of any that have been considered previously.

McCarthy and Cerecedo[16] place the vitamin requirement of the mouse at 1 I.U. per animal per day.

3. Rabbits

Although it is recognized that the rabbit requires vitamin A, few quantitative data have been obtained on this species. Phillips and Bohstedt[17] have reported that rabbits fed a vitamin A-free diet can be maintained in a satisfactory state of health when supplemented with carotene at a level equivalent to 83 I.U. of vitamin A per kilogram of body weight per day.

4. Dogs

Based on the rate of disappearance from the liver, the vitamin A requirement of the dog has been estimated as being between 23 and 47 I.U. per kilogram of body weight per day.[18]

5. Foxes and Minks

One of the characteristics of vitamin A deficiency in the fox is the development of certain nervous symptoms. Using this syndrome as a criterion, Smith[19] placed the minimum daily requirement of the fox at from 15 to 25 I.U. per kilogram of body weight. Storage of the vitamin did not take place until the intake of the vitamin was increased to 50 to 100 I.U. Bassett *et al.*[20] found 25 I.U. per kilogram of body weight per day to be the minimal level which would allow satisfactory growth and a detectable content of vitamin A in the blood serum. The requirement of the mink, although not exactly established, was estimated by the same workers as being somewhat less than that of the fox.

6. Horses

The horse was one of the species used in the original work from which the concept of the relationship between body size and vitamin A requirement developed. Using nyctalopia as a criterion, the requirement of the horse was

[16] P. McCarthy and L. R. Cerecedo, *Federation Proc.* **9**, 364 (1950).
[17] P. H. Phillips and G. J. Bohstedt, *J. Nutrition* **15**, 309 (1938).
[18] P. D. Crimm and D. M. Short, *Am. J. Physiol.* **118**, 477 (1937).
[19] S. E. Smith, *J. Nutrition* **24**, 97 (1942).
[20] C. F. Bassett, J. K. Loosli, and F. Wilke, *J. Nutrition* **35**, 629 (1948).

placed at 17 to 22 I.U. per kilogram of body weight per day when vitamin A was fed, or 20 to 30 γ when carotene was fed.[2] Since this level is probably not adequate for reproduction, the NRC subcommittee reporting on this animal recommended an intake of 180 I.U. of vitamin A as carotene per kilogram of body weight per day.[21] It was the opinion of this committee that this level is probably adequate also for the mule.

7. Swine

The daily vitamin A intake which is sufficient to prevent any detectable sign of nyctalopia in swine is reported as being from 18 to 24 I.U. of vitamin A or 25 to 39 γ of carotene per kilogram of body weight.[2] This level probably does not allow for adequate storage or for the demands of other functions. The recommendation of the NRC subcommittee was 150 I.U. of vitamin A as carotene per kilogram of body weight per day for growing animals, 200 units for pregnant animals, and 330 units for lactating females.[22]

8. Cattle

Cattle have been intensively studied with respect to their requirement for vitamin A. The results of some of these studies are summarized in Table XIX. Depending on the standard used, the requirement has been placed at 25 to 150 I.U. per kilogram of body weight. The 220 I.U. per kilogram of body weight per day as recommended by the subcommittees on beef cattle and dairy cattle of the NRC are such as to meet the exigencies of practice and hence contain a considerable margin of safety. To meet the extra requirements of the last two months before parturition a daily intake of 30 mg. of carotene is recommended.[22] Such a feeding practice will assure a high vitamin A level in the colostrum.

9. Sheep

The daily intake of vitamin A which is sufficient to prevent nyctalopia in sheep is reported as 17 to 26 I.U. of vitamin A or 25 to 35 γ of carotene.[2] The recommendation of the NRC subcommittee which should allow for adequate storage and meet the demands of reproduction and lactation was

[21] P. B. Pearson, C. F. Winchester, and A. L. Harvey, Recommended Nutrient Allowances for Horses, National Research Council (U. S.), Washington, D. C., 1949.

[22] E. H. Hughes, W. M. Beeson, E. W. Crampton, and N. R. Ellis, Recommended Nutrient Allowances for Swine, National Research Council (U. S.), Washington, D. C., 1950.

TABLE XIX

ESTIMATES OF THE VITAMIN A REQUIREMENT OF CATTLE

I.U. fed per kilogram of body weight per day	Criterion used	References
32	Growth, nyctalopia	23
44	Growth	24
43–55	Nyctalopia	2
60	Growth	25
64	Growth	23
83	Fertility in bulls	26
100	Protection for winter conditions	24
110	Spinal fluid pressure in Holstein calves	27
115	Spinal fluid pressure in Jersey calves	27
125	Spinal fluid pressure in Guernsey calves	27
125	Blood level in Holsteins	28
165	Reproduction	29
210	Blood level in Guernseys	28
220	Exigencies of normal conditions	27
250	Growth, liver storage	23
220	NRC recommendation	30, 31

TABLE XX

ESTIMATES OF THE VITAMIN A REQUIREMENT OF CHICKENS

I.U. fed per pound of diet	Criterion used	References
	Starting and growing chicks	
700	Growth	33
1100	Growth, survival, liver storage	34
1000	Growth	35
1200	Growth	36
1400	Growth	37
1400	Growth, general health	38
1600	Growth, liver storage	39
1900	Liver storage	40
2000	NRC recommendation	41
	Laying and breeding hens	
2000	Mortality, egg production, hatchability	42
2000	Hatchability, chick survival	43
2100	Egg production, hatchability	33
2300	Maintenance of laying hens	35
3200	Maintenance of breeding hens	35
4200	Liver storage, egg production, survival, hatchability	44
4200	Egg production, fertility, hatchability	38
3300	NRC recommendation	41

placed at 660 to 930 I.U. of vitamin A, as carotene, per kilogram of body weight. The growth requirement was estimated as 220 units.[32]

10. Chickens

The requirements of both growing chicks and laying hens have been intensively investigated. The criteria used, and the intake recommended by several investigators, are summarized in Table XX. With a few exceptions, the levels recommended have been quite consistent for a particular criterion. Under proper feeding conditions, the levels recommended by the NRC subcommittee, 2000 I.U. per pound of feed for growing chicks and 3300 I.U. for laying and breeding hens, should prove adequate.

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[23] J. M. Lewis and L. T. Wilson, *J. Nutrition* **30,** 467 (1945).

[24] H. A. Keener, S. I. Bechdel, N. B. Guerrant, and W. T. S. Thorp, *J. Dairy Sci.* **25,** 571 (1942).

[25] J. H. Jones, J. K. Riggs, G. S. Fraps, J. M. Jones, H. Schmidt, R. E. Dickson, P. E. Howe, and W. H. Black, *Proc. Am. Soc. Animal Production* **31,** 94 (1938).

[26] L. L. Madsen, O. N. Eaton, L. Heemstra, R. E. Davis, C. A. Cabell, and B. Knapp, *J. Animal Sci.* **7,** 60 (1948).

[27] L. A. Moore, J. F. Sykes, W. C. Jacobson, and H. G. Wiseman, *J. Dairy Sci.* **31,** 533 (1948).

[28] P. D. Boyer, P. H. Phillips, N. S. Lundquist, C. W. Jensen, and I. W. Rupel, *J. Dairy Sci.* **25,** 433 (1942).

[29] A. H. Kuhlman and W. D. Gallup, *J. Dairy Sci.* **25,** 688 (1942).

[30] J. K. Loosli, C. F. Huffman, W. E. Petersen, and P. H. Phillips, Recommended Nutrient Allowances for Dairy Cattle, National Research Council (U. S.), Washington, D. C., 1950.

[31] H. R. Guilbert, P. Gerlaugh, and L. L. Madsen, Recommended Nutrient Allowances for Beef Cattle, National Research Council (U. S.), Washington, D. C., 1950.

[32] P. B. Pearson, W. G. Kammlade, J. I. Miller, and R. F. Miller, Recommended Nutrient Allowances for Sheep, National Research Council (U. S.), Washington, D. C., 1949.

[33] H. J. Almquist, T. H. Jukes, and W. E. Newlon, *Calif. Agr. Extension Circ.* **108** (September 1940).

[34] G. E. Bearse and M. W. Miller, *Poultry Sci.* **16,** 34 (1937).

[35] C. F. McClary, V. L. Miller, and G. E. Bearse, *Western Wash. Expt. Sta. Bull.* (December 1942).

[36] M. W. Taylor and W. C. Russell, *Poultry Sci.* **26,** 234 (1947).

[37] R. E. Gray and H. E. Robinson, *Poultry Sci.* **20,** 36 (1941).

[38] R. M. Sherwood and G. S. Fraps, *Texas Agr. Expt. Sta. Bull.* **583,** (1940).

[39] E. L. Johnson, C. W. Carrick, and S. M. Hauge, *Poultry Sci.* **27,** 308 (1948).

[40] D. W. Bolin, C. E. Lampman, and L. R. Berg, *Poultry Sci.* **22,** 348 (1943).

[41] W. W. Cravens, H. J. Almquist, R. M. Bethke, H. R. Bird, and L. C. Norris, Recommended Nutrient Allowances for Poultry, National Research Council (U. S.), Washington, D. C., 1950.

[42] M. W. Taylor, J. R. Stern, and W. C. Russell, *Poultry Sci.* **26,** 243 (1947).

[43] H. J. Almquist and E. Mecchi, *Poultry Sci.* **18,** 129 (1939).

[44] M. Rubin and H. R. Bird, *Maryland Agr. Expt. Sta. Bull.* **A-12** (1942).

11. Turkeys

Studies on the vitamin A requirement of turkeys have been limited mainly to poults. The results of several of these studies are summarized in Table XXI. The NRC subcommittee recommendation, 4000 I.U. per pound of feed, applies to both poults and breeders and is therefore in excess of what is required for growth. A level of 2500 I.U. per pound of feed should be adequate for growth.

12. Insects

A few species of insects are the only invertebrates that have been studied specifically as to their vitamin A requirements. None of the insects studied

TABLE XXI

Estimates of the Vitamin A Requirements of Turkey Poults

I.U. fed per pound of diet	Criterion used	References
1400	Growth	45
1400	Growth	46
1900	Growth, survival	47
2300	Gross evidence of deficiency	45
2300	Gross evidence of deficiency	46
2700	Growth, survival	48
5400	Blood level, liver storage	49
7700	Growth	49
4000	NRC recommendation for poults and breeders	41

(the cockroach, *Blatella germanica*,[50] the clothes moth, *Tineola bisselliella*,[51] the flour beetle, *Tribolium confusum*[52]) were shown to require vitamin A or carotene. In the cockroach the body lipids were shown to be free of vitamin A.[50] The failure to demonstrate a need for vitamin A by these species and the frequent use of media that are essentially free of vitamin A and carotene for raising invertebrates probably indicates that they do not require vitamin A.

[45] W. C. Russell, M. W. Taylor, and R. Van Reen, *Federation Proc.* **8**, 394 (1949).
[46] R. Van Reen, M. W. Taylor, and W. C. Russell, *J. Nutrition* **43**, 235 (1951).
[47] H. M. Scott, *Kansas Agr. Expt. Sta. Bull.* **276**, (1937).
[48] H. S. Wilgus, *Colo. Farm. Bull.* **2**, 3 (1940).
[49] F. D. Wharton, L. D. Matterson, H. M. Scott, and C. I. Bliss, *J. Nutrition* **39**, 543 (1949).
[50] R. E. Bowers and C. M. McCay, *Science* **92**, 291 (1940).
[51] M. F. Crowell and C. M. McCay, *Physiol. Zoöl.* **10**, 368 (1937).
[52] M. D. Sweetman and L. S. Palmer, *J. Biol. Chem.* **77**, 33 (1928).

B. OF HUMAN BEINGS

KARL E. MASON

1. The International Unit

Dosage and requirements of vitamin A are usually expressed in terms of international units (I.U.). This unit has a constant value; the standard of reference for the unit, against which commercial preparations are assessed, also has a constant biological value, although it has varied chemically as more suitable compounds have become available. The 1931 reference standard of 1 γ of crystalline carotenes was replaced in 1934 by 0.6 γ of crystalline β-carotene, and in 1949 by 0.344 γ of vitamin A acetate (or 0.3 γ of vitamin A alcohol) which had previously been adopted by the United States Pharmacopeia to replace the older U.S.P. reference cod liver oil standard. The 1934 reference standard of 0.6 γ of β-carotene is retained for the measurement of carotenes. As implied in these standards and as borne out in biological tests, the C_{40} molecule of carotene produces but one C_{20} molecule of vitamin A.

Vitamin A in dietary sources and in fish liver oils and concentrates exists chiefly as natural esters which are hydrolyzed by pancreatic lipase and absorbed as the less stable vitamin A alcohol, which is re-esterified in the intestinal wall. The presence of fat and bile salts is necessary for adequate absorption of both vitamin A and carotene. Concepts of the subsequent fate of vitamin A ester and carotene have been considerably modified by recent experiments on a variety of animals. This subject has been carefully reviewed by Kon and Thompson.[53] If these finding are applicable to man, we must visualize the intestinal wall as the major site of conversion of carotene to vitamin A, rather than the liver as was once believed, and the lymphatic stream rather than the portal vein as the major pathway of transport of these substances from the intestine to the general circulation from which the liver and other tissues, under normal conditions, remove the excess and maintain a reasonably constant blood level of carotene (about 120 I.U. per 100 ml.) and of vitamin A (about 75 to 100 I.U. per milliliter, predominantly as vitamin A alcohol). It is still undetermined whether much of the carotene of the circulating blood or of that stored in various tissues can be converted into vitamin A.

2. Requirements for Carotene

The most valuable evidence concerning the requirements of young adults for carotene, when this represents the sole cource of vitamin A, comes

[53] S. K. Kon and S. Y. Thompson, *Brit. J. Nutrition* **5**, 114 (1951).

from the Sheffield experiment,[54] where prophylactic and curative tests on human volunteers on a diet essentially devoid of vitamin A and carotene indicated a minimal daily requirement of 1500 I.U. of β-carotene, assuming that all the carotene administered was absorbed. To allow for individual variation and for a certain margin of safety, the original estimate was doubled. The value of 3000 I.U. required further modification to allow for incompleteness of absorption, which has long been recognized as varying considerably with the source of the carotene. For instance, in these studies it was found that fecal excretion of carotene was of such magnitude as to require a daily intake of 4000 I.U. of pure β-carotene in oil, 5000 I.U. of carotene as homogenized carrots, 7500 I.U. as cabbage or spinach, and 12,000 I.U. as boiled sliced carrots (as measured chemically), in order to assure absorption of the estimated requirement of 3000 I.U. Consequently, as explained in an excellent review of the standardization of vitamin A and human requirements by Hume,[55] a compromise value of 7500 I.U., or three times the recommended vitamin A requirement, was somewhat reluctantly proposed as the daily requirement if provided solely as carotene from average dietary sources.

3. Requirements for Vitamins A

Human requirements have been estimated on the general assumption that only about two-thirds of the vitamin A of the average diet is provided as carotenoid precursors, and the remaining as preformed vitamin A. Estimation of these requirements has been guided by (1) experiences in determining daily needs for preventing impaired dark adaptation in human volunteers on controlled low intakes of vitamin A, (2) measurements of vitamin A storage in the liver and other tissues from cases of accidental death in healthy individuals, and (3) the estimation of carotene and vitamin A in the diet of well-nourished individuals. In utilizing data from the therapeutic tests, consideration has been given to the fact that an optimal intake should represent two to four times the minimal intake required to prevent manifestations of the deficiency state. In these computations consideration has also been given to the extent to which vitamin A needs may be modified by age, rate of growth, caloric intake, physical expenditure and special physiological needs such as arise during pregnancy and lactation. The daily dietary allowances for vitamin A, as recommended by the Food and Nutrition Board of the National Research Council in its 1948 report,[56] are

[54] E. M. Hume and H. A. Krebs, *Med. Research Council Brit. Spec. Rept. Ser.* **264** (1949).

[55] E. M. Hume, *Brit. J. Nutrition*, **5**, 104 (1951).

[56] *Bull. Natl. Research Council (U. S.), Reprint and Circ. Ser.* **129** (1948).

as follows:

TABLE XXII

RECOMMENDED DAILY DIETARY ALLOWANCES OF VITAMIN A[a]

Children		Adults	
1 year	1500 I.U.	Men (70 kg.)	
1–3 years	2000 I.U.	Sedentary	5000 I.U.
4–6 years	2500 I.U.	Physically active	5000 I.U.
7–9 years	3500 I.U.	Heavy work	5000 I.U.
10–12 years	4500 I.U.		
13–15 years		Women (56 kg.)	
Girls	5000 I.U.	Sedentary	5000 I.U.
Boys	5000 I.U.	Mod.active	5000 I.U.
16–20		Very active	5000 I.U.
Girls	5000 I.U.	Pregnancy	
Boys	6000 I.U.	(latter half)	6000 I.U.
		Lactation	8000 I.U.

[a] Based on the assumption that two-thirds of the intake is provided as carotene, and that carotene has one-half or less than one-half the biological value of vitamin A.

The recommended allowance for the average adult (5000 I.U. from combined sources) represents the equivalent of about 3250 I.U. of vitamin A, or 6500 I.U. of carotene, if either provided the entire requirement. This is in reasonable accord with the results of the Sheffield experiment,[54] on the basis of which 2500 I.U. of vitamin A or 7500 I.U. of carotene were the recommended levels of requirement for young adults. The recommended daily requirements are at least twice the minimal needs to prevent any manifestation of avitaminosis A. The therapeutic requirements to resolve clinical evidence of avitaminosis A are usually accepted as at least twenty times the minimal need, i.e., about 50,000 I.U.

All forms of vitamin A are absorbed well by healthy individuals, but natural vitamin A esters are not as effectively absorbed as are finely emulsified esters or the alcohol form in oil or in aqueous dispersion. These modified forms of the vitamin have greater therapeutic value than the unemulsified ester where intestinal absorption is below par.[57, 58] Since approximately 99 % of vitamin A in the diet and in fish liver oil preparations is in the form of natural esters, the factor of differential absorption of different forms of the vitamin requires little consideration in meeting requirements for vitamin A in the normal individual.

[57] A. B. McCoord, C. P. Katsampes, C. F. Lavender, F. J. Martin, R. A. Ulstrom, and R. H. Tully, *Pediatrics* **2**, 652 (1948).

[58] C. D. May and C. U. Lowe, *J. Clin. Invest.* **27**, 226 (1948).

4. Features Influencing Requirements

The factors which may modify individual needs for vitamin A fall generally into three categories. First, conditions which interfere with the normal absorption of vitamin A and with the absorption and intestinal conversion of carotene to vitamin A. Second, types of liver injury which alter the capacity of the liver to store or to regulate the release of vitamin A. Third, changes in the general metabolism of the individual which result in excessive utilization or excretion of vitamin A reserves.

a. Factors Interfering with Absorption

Inadequacy of pancreatic lipase for hydrolysis of vitamin A esters and of bile salts, and conditions which alter the normal function and structure of the small intestine, seriously impair the absorption of vitamin A and carotene. For these reasons, requirements for vitamin A are increased in premature infants, and in disease states such as pancreatic fibrosis, celiac disease, sprue, infectious hepatitis, congenital obstruction of the bile ducts, giardiasis, prolonged diarrhea, colitis, and generalized infections.[57-60] There is evidence of diminished intestinal absorption of vitamin A and carotene in elderly individuals.[61] The presence of mineral oil interferes with the absorption of carotene, and to a lesser extent with the absorption of vitamin A. Although symptoms of avitaminosis A have been observed in infants with congenital biliary atresia[62] and giardiasis,[63] the above-mentioned conditions usually do not interfere to the extent of causing outspoken manifestations of the deficiency state.

b. Hepatic Dysfunctions

Cirrhosis and other types of injury to the liver alter the capacity of this organ to store vitamin A, to convert vitamin A esters to vitamin A alcohol, or to release the latter in normal amounts to the circulating plasma. There is also evidence that a great variety of fatal diseases alter the normal functions of the liver in the metabolism of vitamin A. Of particular interest in this connection are the analyses of vitamin A reserves in the liver of 745 human subjects carried out by Moore and Sharman,[64] leading to the conclusion that "in most fatal diseases at least half the vitamin A reserves disappear"; only in thyroid disease and diabetes were liver reserves higher

[59] S. W. Clausen, *Harvey Lectures* **38**, 199 (1943).
[60] S. Spector, C. F. McKhann, and E. R. Meserve, *Am. J. Diseases Children* **66**, 376 (1943).
[61] H. A. Rafsky and B. Newman, *Gastroenterology* **10**, 1001 (1948).
[62] M. D. Altschule, *Arch. Pathol.* **20**, 845 (1935).
[63] P. V. Veghelyi and F. J. Lancos, *Am. J. Diseases Children* **78**, 257 (1949).
[64] T. Moore and I. M. Sharman, *Brit. J. Nutrition* **5**, 119 (1951).

than in cases of accidental death. To what extent the lowered reserves are due to low intake, defective absorption and conversion, or increased destruction remains to be determined.

c. Metabolic Influences

During pregnancy and lactation there are increased requirements to meet the metabolic needs of the mother and to provide for an adequate transfer of vitamin A to the fetus and to the mother's milk. Artificial hyperthermia and febrile states in general reduce plasma levels of vitamin A, owing to a variable degree to excessive utilization, inhibited release from the liver and impaired absorption, causing at least a temporary increase in requirements. Whether tocopherols can enhance liver storage of vitamin A in man, as they have been shown to do in experimental animals, is not known. They are reported as having little or no beneficial effect on the absorption of various forms of vitamin A in man.[65] There are suggestions that sex differences in the utilization of vitamin A may exist in man,[65] a phenomenon which is quite marked in experimental animals.[64]

d. Kidney Dysfunctions

There is evidence, recently summarized by Moore and Sharman,[64] that vitamin A, which under normal conditions is not excreted by the human kidney, appears in considerable amounts in the urine of patients with chronic nephritis, pneumonia, and icterus. A somewhat antithetical situation exists in the nephrotic syndrome in children, where serum vitamin A levels are unusually high but there is no evidence of urinary loss.[66] Following administration of test doses of vitamin A the serum levels show a marked and sustained elevation. This is attributed to a failure of the liver to utilize or store vitamin A due to depletion of a liver protein component to which vitamin A becomes attached.

[65] E. F. Week and F. J. Sevigne, *J. Nutrition* **40**, 563 (1950).

[66] B. M. Kagan, E. M. Thomas, D. A. Jordan, and A. F. Abt, *J. Clin. Invest.* **29**, 141 (1950).

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Chapter 2

ASCORBIC ACID

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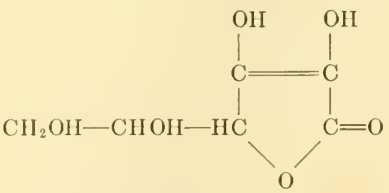
I. Nomenclature and Formulas

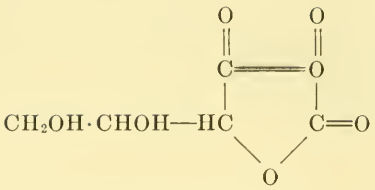
ROBERT S. HARRIS

Accepted names: Ascorbic acid[1]
Vitamin C[2]
Obsolete names: Cevitamic acid
Hexuronic acid[3]
Antiskorbutin[4]
Antiscorbutic vitamin
Scorbutamin[5]
Empirical formulas: $C_6H_8O_6$ (ascorbic acid)
$C_6H_6O_6$ (dehydroascorbic acid)
Chemical name: 1-Threo-2,4,5,6-pentoxyhexen-2 carboxylic acid lactone
Structures:



Ascorbic acid
(reduced form)

[1] A. Szent-Györgyi and W. N. Haworth, *Nature* **131**, 23 (1933).
[2] J. C. Drummond, *Biochem. J.* **14**, 660 (1920).
[3] A. Szent-Györgyi, *Biochem. J.* **20**, 1387 (1925).
[4] A. Holst, and T. Frölich, *J. Hyg.* **7**, 634 (1907).
[5] R. L. Jones, *Science* **68**, 480 (1928).



Dehydroascorbic acid
(oxidized form)

II. Chemistry

FRED SMITH

A. ISOLATION OF L-ASCORBIC ACID

In the seventeenth century it was known that sailors at sea for long periods frequently developed the disease called scurvy, and as far back as the middle of the eighteenth century this was traced to the fact that fresh fruit and vegetables were not included in the diet. Furthermore, it was established that lemon juice was an excellent remedy for the disease. At that time, of course, nothing was known of accessory food factors or vitamins; and little further progress took place until it was found that guinea pigs were also subject to the disease of scurvy as well as man[1-3] Thereafter it became possible to evaluate the antiscorbutic power of various natural products,[4] and controlled experiments with these animals provided the necessary means of tracing the active antiscorbutic principle during the early attempts at its concentration.

Much of the early and, consequently, the most difficult work on the concentration of the antiscorbutic factor or vitamin C was carried out by Zilva, who was endeavoring to isolate the active principle from lemons.[5, 6] After removal of the greater proportion of extraneous material such as fiber and citric acid, the active constituent was precipitated by adding basic lead acetate, a procedure used today in the isolation of vitamin C from natural products. The lead complex was then decomposed with hydrogen sulfide, and upon evaporation of the solution there was obtained a sirupy product which showed a high degree of antiscorbutic activity. Although such con-

[1] A. Holst, *J. Hyg.* **7**, 619 (1907).
[2] A. Holst and T. Fröhlich, *J. Hyg.* **7**, 634 (1907).
[3] A. Holst and T. Fröhlich, *Z. Hyg.* **72**, 1 (1912).
[4] H. Chick and E. M. Hume, *Trans. Roy. Soc. Trop. Med. Hyg.* **10**, 141 (1916–1917); *Proc. Roy. Soc. (London)* **B90**, 44 (1917–1919).
[5] A. Harden and S. S. Zilva, *Biochem. J.* **12**, 259 (1918).
[6] S. S. Zilva, *Biochem. J.* **17**, 416 (1923); **18**, 186 (1924); **19**, 589 (1925).

centrated samples of vitamin C were found to vary in their activity, an observation which we can well understand, bearing in mind the labile nature of vitamin C, some of them were so active that it seems surprising that they did not crystallize. At that time such samples could not be used by the organic chemist for precise chemical investigations, but examination of them enabled Zilva to establish the general properties of the vitamin. It was clear from his investigations that the active principle was water-soluble and that it was an organic substance with extremely strong reducing properties which did not contain nitrogen. It was also recognized that the active principle was probably related to the sugars and that solutions of it readily decomposed when exposed to air and especially in the presence of alkali.[7] In making use of phenolindophenol to determine the reducing power of vitamin C concentrates, Zilva also made the significant observation that the physiological activity was proportional to the reducing power. He made the further discovery that freshly oxidized solutions (which are now known to contain dehydroascorbic acid) still retained their activity. It was this fact which led Zilva to point out that the reducing and antiscorbutic properties might not arise from one and the same substance.[8] This apparent anomaly was resolved by Tillmans, who was also interested in the relationship between the antiscorbutic principle and the reducing factor. He correctly concluded that the vitamin could be reversibly oxidized and that both the oxidized and the reduced forms were physiologically active.[9-11]

While the later stages of this work had been going on, Szent-Györgyi, in investigating oxidation-reduction systems in plants and animals, succeeded in isolating from orange juice, from cabbage juice, from the adrenal glands of oxen, and later from paparika a crystalline optically active substance with the formula $C_6H_8O_6$. The substance was acidic in nature, it exhibited very strong reducing properties, and it gave color tests characteristic of the sugars. For these reasons he named it "hexuronic acid."[12, 13] The isolation by King shortly thereafter of an antiscorbutically active crystalline substance from lemon juice,[14] coupled with the demonstration by

[7] S. J. B. Connell and S. S. Zilva, *Biochem. J.* **18**, 641 (1924).

[8] S. S. Zilva, *Biochem. J.* **21**, 689 (1927).

[9] J. Tillmans, *Z. Untersuch. Lebensm.* **60**, 34 (1930).

[10] J. Tillmans, P. Hirsch, and W. Hirsch, *Z. Untersuch. Lebensm.* **63**, 1 (1932); J. Tillmans, P. Hirsch, and F. Siebert, *ibid.* **63**, 21 (1932); J. Tillmans, P. Hirsch, and J. Jackisch, *ibid.* **63**, 241, 276 (1932); J. Tillmans, P. Hirsch, and H. Dick, *ibid.* **63**, 267 (1932).

[11] J. Tillmans, P. Hirsch, and R. Vaubel, *Z. Untersuch. Lebensm.* **65**, 145 (1933).

[12] A. Szent-Györgyi, *Biochem. J.* **22**, 1387 (1928).

[13] J. L. Svirbely and A. Szent-Györgyi, *Biochem. J.* **27**, 279 (1933).

[14] W. A. Waugh and C. G. King, *J. Biol. Chem.* **97**, 325 (1932).

Szent-Györgyi that his hexuronic acid was also antiscorbutically active,[11, 15-18] strongly suggested that these crystalline materials were in fact vitamin C, a conclusion which was in line with a suggestion to this effect made previously by Tillmans from a series of investigations on vitamin C.[10, 11] However, there was still some doubt about this conclusion, because relatively large doses of the so-called pure crystalline hexuronic acid were required to cure scurvy whereas with other deficiency diseases very much smaller doses of the appropriate vitamin or vitamin concentrates were already known to be capable of effecting a cure. This gave support to the view that the active principle was present in the crystalline hexuronic acid as a contaminant no matter how many times it was recrystallized. Against this view, however, could be advanced the observation that all the samples of hexuronic acid, irrespective of their source, had the same antiscorbutic activity. It was further shown that, whereas the crystalline monoisopropylidene derivative of hexuronic acid was inactive, the hexuronic acid regenerated from it by hydrolysis had the same antiscorbutic activity as the original material from which the isopropylidene derivative was prepared.[13] All doubts about hexuronic acid's being the true vitamin were finally removed when it was found that synthetic hexuronic acid, or ascorbic acid as it was later called, had exactly the same physiological activity as the hexuronic acid isolated from natural sources.[19, 20]

B. NATURAL OCCURRENCE OF L-ASCORBIC ACID

Since the first isolation of this crystalline vitamin in 1928,[12] a great deal of work has been done in determining the ascorbic acid content of many foods, fruits, vegetables, and plants, and it is now apparent that ascorbic acid is widely distributed in the plant and animal kingdoms. The paprika plant (*Capsicum* sp.) found by Szent-Györgyi to be an excellent source of the vitamin[13] is now rivaled by such sources as rose hips (*Rosa* sp.),[11, 21, 22] pine needles,[22] sea buckthorn berries (*Hippophaé rhamnoides*),[23] and guava (*Psidium guayava* L.).[24] A search for alternative sources of ascorbic acid

[15] J. L. Svirbely and A. Szent-Györgyi, *Nature* **129**, 576 (1932).
[16] J. L. Svirbely and A. Szent-Györgyi, *Nature* **129**, 690 (1932).
[17] J. L. Svirbely and A. Szent-Györgyi, *Biochem. J.* **26**, 865 (1932).
[18] T. W. Birch, L. J. Harris, and S. N. Ray, *Nature* **131**, 273 (1933).
[19] T. Reichstein, A. Grüssner, and R. Oppenauer, *Helv. Chim. Acta* **16**, 1019 (1933).
[20] W. N. Haworth, E. L. Hirst, and S. S. Zilva, *J. Chem. Soc.* **1934**, 1155.
[21] G. Hunter and J. Tuba, *Can. Med. Assoc. J.* **48**, 30 (1943); C. A. Kepkova, *Compt. rend. acad. sci. U.R.S.S.* **48**, 655 (1945)[*C.A.* **40**, 6124 (1946)].
[22] O. Shnaidman, *Pishchevaya Prom.* **1943**, No. 1/2, 5 [*C.A.* **40**, 113 (1946)].
[23] F. Brühne, *Deut. Apoth. Ztg.* **58**, No. 61/62; *Deut. Heilpflanze* **45** (1943) [*C.A.* **39**, 2585 (1945)]; A. Jermstad and E. Brockmann-Hansen, *Medd. Norsk Farm. Selskap* **6**, 86 (1944) [*C.A.* **41**, 7563 (1947)].
[24] H. E. Munsell, *Food Research* **10**, 42 (1945).

became intensified during World War II because the import of citrus fruits into Europe and Asia was stopped and also because there was an inadequate supply of synthetic ascorbic acid to provide the dietary requirement. Still more recently unripe walnuts (ascorbic acid content 0.6 to 1.0%)[22, 25] and the West Indian cherry (*Malpighia punicifolia*) have been found to contain relatively large amounts of ascorbic acid (1.0 to 1.4%).[26] It appears that the West Indian cherry is the richest fruit source of ascorbic acid so far discovered.[27]

It is of interest to compare the difficulties encountered by Zilva, Szent-Györgyi, and King in their early attempts to concentrate and crystallize ascorbic acid before its labile chemical nature was known, with the ease with which it can be isolated in the pure crystalline form from natural sources now that the chemistry of the substance is known and the appropriate precautions are taken to prevent decomposition (e.g., see procedure given in ref. 25).

C. CHEMICAL NATURE AND CONSTITUTION OF L-ASCORBIC ACID

Vitamin C or hexuronic acid is a white crystalline compound (formula $C_6H_8O_6$) with a melting point of 192° and a specific rotation in water of $[\alpha]_D + 23°$. It reacts as a monobasic acid and liberates carbon dioxide from carbonates and bicarbonates to form well-defined salts with the general formula $C_8H_7O_6M$. Vitamin C is a very powerful reducing substance, being much more strongly reducing than the normal sugars as indicated by its ability to reduce Fehling's solution and Tollens' ammoniacal silver nitrate at room temperature.[12] Its reducing action on silver and gold salts to give the metals is so effective that the reaction has been recommended as an analytical method for the determination of gold[28] and silver[29] and also for determining vitamin C concentrations in certain natural products.[30] It displays many of the color reactions shown by carbohydrates, probably because upon treatment with rather strong acids it gives furfuraldehyde.[10] The formation of the latter and the fact that upon reduction ascorbic acid gives L-idonic acid[31] show that the six carbon atoms in the vitamin molecule form a straight chain. Ascorbic acid shows the remarkable property of reacting immediately in the cold with two equivalent proportions of the

[25] A. A. Klose, J. B. Stark, G. G. Purvis, J. Peat, and H. L. Fevold, *Ind. Eng. Chem.* **42**, 387 (1950).
[26] C. F. Asenjo and A. R. Freire de Guzmán, *Science* **103**, 219 (1946).
[27] C. F. Asenjo and C. G. Moscoso, *Food Research* **15**, 103 (1950).
[28] E. C. Stathis and H. C. Gatos, *Ind. Eng. Chem. Anal. Ed.* **18**, 801 (1946).
[29] E. C. Stathis, *Anal. Chem.* **20**, 271 (1948).
[30] C. Griebel and M. Rohkohl, *Z. Lebensm. Untersuch. u. Forsch.* **88**, 2 (1948).
[31] F. Micheel and K. Kraft, *Hoppe-Seyler's Z. physiol. Chem.* **218**, 280 (1933).

halogens chlorine, bromine, or iodine in neutral or in acid solution.[32] This reaction with the halogens, which does not proceed in non-aqueous solvents unless a carbonate such as lead carbonate is present to remove the acid, is one of oxidation and not of addition of halogen, for two equivalents of halogen acid are liberated. Thus two atoms of hydrogen are removed from each mole of ascorbic acid ($C_6H_8O_6$) to give dehydroascorbic acid ($C_6H_6O_6$). The oxidation reaction is a reversible one, for if the oxidized solutions are treated with hydrogen sulfide the original vitamin is regenerated.[33] If iodine has been used as the oxidizing agent, reversal of the reaction can be effected by simply evaporating the oxidized solution *in vacuo*. The hydriodic acid acts as the reducing agent, and iodine and ascorbic acid are formed. The same well-defined oxidation of ascorbic acid can be carried out with a large number of oxidizing agents such as acid permanganate,[34] quinone, phenolindophenol[8] and its dichloro derivative,[35] methylene blue,[36] and ferric salts.[37] It is because of this oxidation arrest point that these and other reagents (see p. 248) have been utilized in devising methods for the assay and determination of vitamin C.[38] Dehydroascorbic acid, which is the first oxidation product of ascorbic acid, retains the same antiscorbutic activity as the original vitamin, and it is believed that some of the vitamin C occurring in nature exists in this oxidized form. Aqueous solutions and extracts of ascorbic acid readily undergo oxidation when left exposed to the air, especially if traces of copper are present.[39] In alkaline solutions the oxidation is greatly accelerated and extensive degradation eventually takes place, leading to complete breakdown of the molecule.

The structure of ascorbic acid can be deduced from the following evidence. The compound contains four hydroxyl groups as evidenced by the fact that vigorous acetylation gives rise to a tetraacetate.[40] Two of the hydroxyl groups are different from the others, for under milder acetylation conditions ascorbic acid gives a well-defined diacetate.[41] The two hydroxyl

[32] R. W. Herbert, E. L. Hirst, E. G. V. Percival, R. J. W. Reynolds, and F. Smith, *J. Chem. Soc.* **1933**, 1270.

[33] E. L. Hirst and S. S. Zilva, *Biochem. J.* **27**, 1271 (1933).

[34] K. S. Murty and C. R. Viswanadham, *Proc. Natl. Inst. Sci. India* **10**, 217 (1944).

[35] E. Géro, *Bull. soc. chim. biol.* **31**, 817, 825 (1949); J. Tillmans, *Z. Untersuch. Lebensm.* **54**, 33 (1927); L. P. Pepkowitz, *J. Biol. Chem.* **151**, 405 (1943); J. W. H. Lugg, *Australian J. Exptl. Biol. Med. Sci.* **20**, No. 4, 273 (1942).

[36] F. S. Trucco, *Ann. chim. appl.* **34**, 127 (1944).

[37] R. Strohecker and E. Sierp, *Z. Lebensm. Untersuch. u. Forsch* **90**, 93 (1950); R. do Nascimento, *Rev. soc. brasil. quím.* **16**, 165 (1947); J. Koch and W. Diemar, *Pharmazie* **3**, 104 (1948).

[38] D. G. Chapman, O. Rochon, and J. A. Campbell, *Anal. Chem.* **23**, 1113 (1951).

[39] R. Strohecker and H. Schmidt, *Z. Lebensm. Untersuch. u. Forsch.* **86**, 370 (1943).

[40] V. A. Devyatnin, *Khim. Referat. Zhur.* **4**, No. 9, 71 (1941) [*C.A.* **38**, 2789 (1944)].

[41] M. Creighton, W. Wenner, and H. M. Wuest, *J. Org. Chem.* **13**, 613 (1948); Ching-Hao Kan, *J. Chinese Chem. Soc.* **17**, 86 (1950).

groups which are acetylated with difficulty display unusual acidic properties, and evidently they are enolic in character inasmuch as they undergo methylation with diazomethane.[42] The methoxyl groups thus introduced are etheric in nature, since they are stable to alkali.[32] The two hydroxyl groups which do not react with diazomethane are of the normal type, since the dimethyl ascorbic acid, prepared with this methylating agent, can be condensed with acetone to give a monoisopropylidene derivative[42, 43] and a di-*p*-nitrobenzoate.[43] It may also be deduced that one of these normal alcoholic groups that react with acetone and *p*-nitrobenzoyl chloride is of the primary type because the dimethyl ascorbic acid can be converted into a trityl compound.[44] The presence of a primary alcohol group in the dimethyl ascorbic acid also follows from the fact that it gives formaldehyde when treated with lead tetraacetate.[31]

Ascorbic acid also shows strong selective absorption of light in the ultraviolet region of the spectrum having a band at $\lambda = 2450$ A. moving to $\lambda = 2650$ A. upon addition of alkali, thus indicating the presence of a conjugated system carrying one or more enolizable hydroxyl groups.[32] That a double bond was present in this conjugated system followed from the fact that catalytic reduction of ascorbic acid adds two atoms of hydrogen, giving L-idonic acid.[31] When ascorbic acid is oxidized with two atomic proportions of iodine, two equivalent proportions of hydrogen are removed to give dehydroascorbic acid. The latter no longer contains a conjugated system, since it shows no selective absorption band in the ultraviolet; but the main structure of the molecule remains intact, for from the dehydro acid the original ascorbic acid may be regenerated by reduction with hydrogen sulfide[32, 33] Further oxidation of the dehydro acid (II or III) with iodine in alkaline solution gives oxalic acid, the latter being readily isolated as its phenylhydrazine salt. Oxidation of ascorbic acid with acid permanganate gives L-threonic acid (IV), recognized by its oxidation with nitric acid to L-tartaric acid (V). The L-threonic acid was also identified by methylation to 2,3,4-trimethyl-L-threonic acid (VI) which formed a crystalline amide.[32]

Of important structural significance was the observation that oxidation of ascorbic acid with chlorine or iodine to give dehydroascorbic acid transformed a strongly acidic substance into an almost neutral substance, the properties of which showed that it was probably a γ-lactone.[45] This accumulated evidence established the fact that ascorbic acid contained a double bond which was located between C_2 and C_3, a deduction previously made

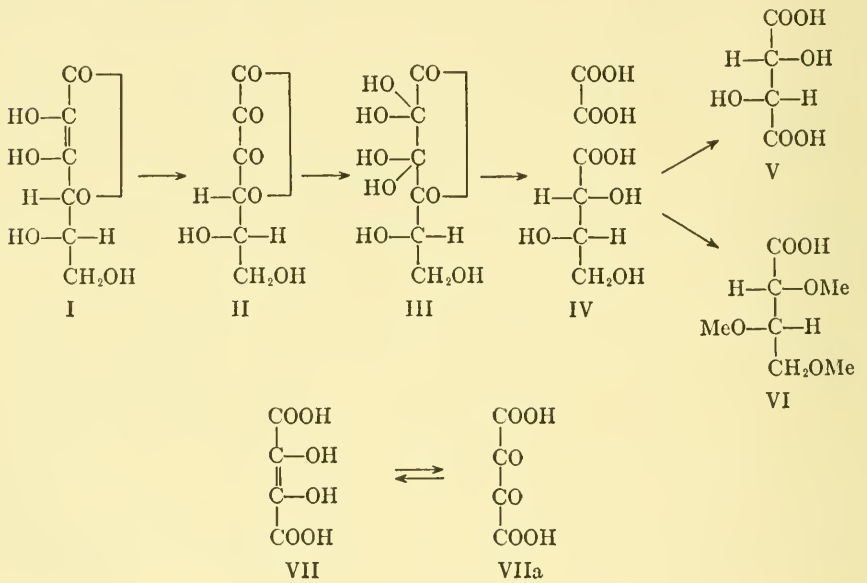
[42] P. Karrer, H. Salomon, K. Schöpp, and R. Morf, *Helv. Chim. Acta* **16**, 181 (1933); P. Karrer, H. Salomon, R. Morf, and K. Schöpp, *Biochem. Z.* **258**, 4 (1933); P. Karrer, G. Schwarzenbach, and K. Schöpp, *Helv. Chim. Acta* **16**, 302 (1933).

[43] F. Micheel and K. Kraft, *Hoppe-Seyler's Z. physiol. Chem.* **215**, 215 (1933).

[44] W. N. Haworth, E. L. Hirst, F. Smith, and W. J. Wilson, *J. Chem. Soc.* **1937**, 829.

[45] E. L. Hirst, *Chemistry & Industry* **52**, 221 (1933).

by Micheel when he demonstrated that the di-*p*-nitrobenzoate of the dimethyl ether of ascorbic acid furnished, upon ozonization, oxalic acid and di-*p*-nitrobenzoyl-L-threonic acid.[43] The acid character was traced to the dienolic system which was present in a ring.[32, 45] Two enolic hydroxyl groups were involved in this system because they disappeared upon oxidation of ascorbic acid to dehydroascorbic acid and they underwent methylation with diazomethane. The two enolic hydroxyl groups located on carbon atoms 2 and 3 do not display the same acidity, for a monomethyl ether could readily be isolated by titration of L-ascorbic acid with ethereal diazomethane.[46, 47] The postulation of formula I for ascorbic acid, showing it



to be a member of the L series, then became possible.[45] Support for this theory was forthcoming from parallel experiments with dihydroxymaleic acid (VII). The latter displayed light absorption properties similar to ascorbic acid, and, moreover, it was found to undergo reversible oxidation with iodine to give the dehydro compound (VIIa) from which VII could be regenerated by reduction with hydrogen sulfide.[32]

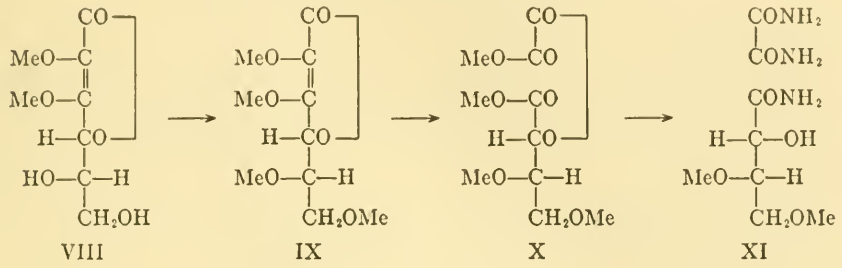
The tentative structure assigned to ascorbic acid (I) and the γ-lactone structure postulated for its first oxidation product, dehydroascorbic acid (II), were also in accord with the fact that the latter readily formed hy-

[46] T. Reichstein, A. Grüssner, and R. Oppenauer, *Helv. Chim. Acta* **17**, 510 (1934); W. N. Haworth and E. L. Hirst, *ibid.* **17**, 520 (1934).

[47] W. N. Haworth, E. L. Hirst, and F. Smith, *J. Chem. Soc.* **1934**, 1556.

drazone-like bodies with *o*-phenylenediamine,[48] phenylhydrazine,[32, 49] and with various substituted phenylhydrazines.[32]

The precise nature of the ring in I, the final point to be established, was proved by the well-known methylation techniques previously developed for the specific purpose of determining the size of the rings in sugars and their derivatives.[50] The neutral 2,3-dimethylascorbic acid (VIII) prepared



from I by methylation with diazomethane was completely methylated with silver oxide and methyl iodide to give the corresponding tetramethyl derivative (IX). The close similarity of the selective absorption bands shown by un-ionized ascorbic acid (I) and its di- (VIII) and tetramethyl ether (IX) proved that no alteration in ring structure took place during these methylation experiments. Cleavage of the double bond in IX was effected with ozone to give the neutral mixed oxalic ester (X). The latter readily reacted with ammonia with the formation of oxamide and the dimethyl-L-threonamide (XI). Isolation of oxamide furnished further proof that a double bond is located between C_2 and C_3, while the characterization of XI as 3,4-dimethyl-L-threonamide by the fact that it gave a positive Weerman test for α-hydroxy amides finally proved that the ring of ascorbic acid was five-membered and engaged C_1 and C_4. The formula (I) assigned to ascorbic acid was thus fully substantiated.[32]

A molecular model of ascorbic acid built on the basis of formula I will be seen to be quite flat. This is in agreement with the x-ray-crystallographic data furnished by Cox and his associates in the early days of the structural investigations into vitamin C.[51] This is of some interest perhaps, because until it was discovered sometime later that dehydro vitamin C was a lactone and not an acid, the x-ray results provided the only evidence which did

[48] H. Ohle and H. Erlbach, *Ber.* **67**, 555 (1934).

[49] H. Ohle and G. Bockmann, *Ber.* **67**, 1750 (1934).

[50] W. N. Haworth, The Constitution of the Sugars, Arnold, London, 1928; E. G. V. Percival, Structural Carbohydrate Chemistry. Prentice-Hall, New York, 1950.

[51] E. G. Cox, *Nature* **130**, 205 (1932); E. G. Cox, and T. H. Goodwin, *J. Chem. Soc.* **1936**, 769; E. G. Cox and E. L. Hirst, *Nature* **131**, 402 (1933).

not agree with the formulation of vitamin C as a derivative of a furancarboxylic acid.[43]

The structure (I) assigned above to L-ascorbic acid received full confirmation from the reactions set out below which have been used for its synthesis.

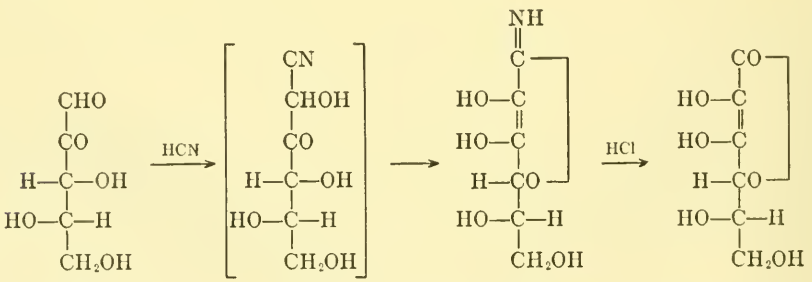
D. SYNTHESIS OF L-ASCORBIC ACID

Four principal methods have so far been devised for the synthesis of L-ascorbic acid and its analogs.

1. *Addition of hydrogen cyanide to a glycosone and conversion of the intermediate iminoascorbic acid into* L-*ascorbic acid by acid hydrolysis.*
2. *Simultaneous lactonization and isomerization of an appropriate 2-keto acid or ester.*
3. *Benzoin condensation of sugars with ethyl glyoxylate or ethyl mesoxalate.*
4. *Claisen condensation of esters of hydroxy acids.*

Method 1. Addition of Hydrogen Cyanide to a Glycosone and Conversion of the Intermediate Iminoascorbic Acid into Ascorbic Acid by Acid Hydrolysis

When an osone such as L-xylosone (XII) is allowed to react with an alkali cyanide in aqueous solution, the usual type of cyanohydrin reaction takes place at C_1. The nitrile (XIII) thus formed but not isolated immediately undergoes ring closure to give the 1-imino derivative[52] of L-ascorbic acid (XIV). The latter already contains the highly reactive enediolic system of ascorbic acid, and upon hydrolysis with dilute acid the desired L-ascorbic acid (I) is readily formed.



XII — L-Xylosone (lyxosone)

XIII

XIV — Imino-L-ascorbic acid

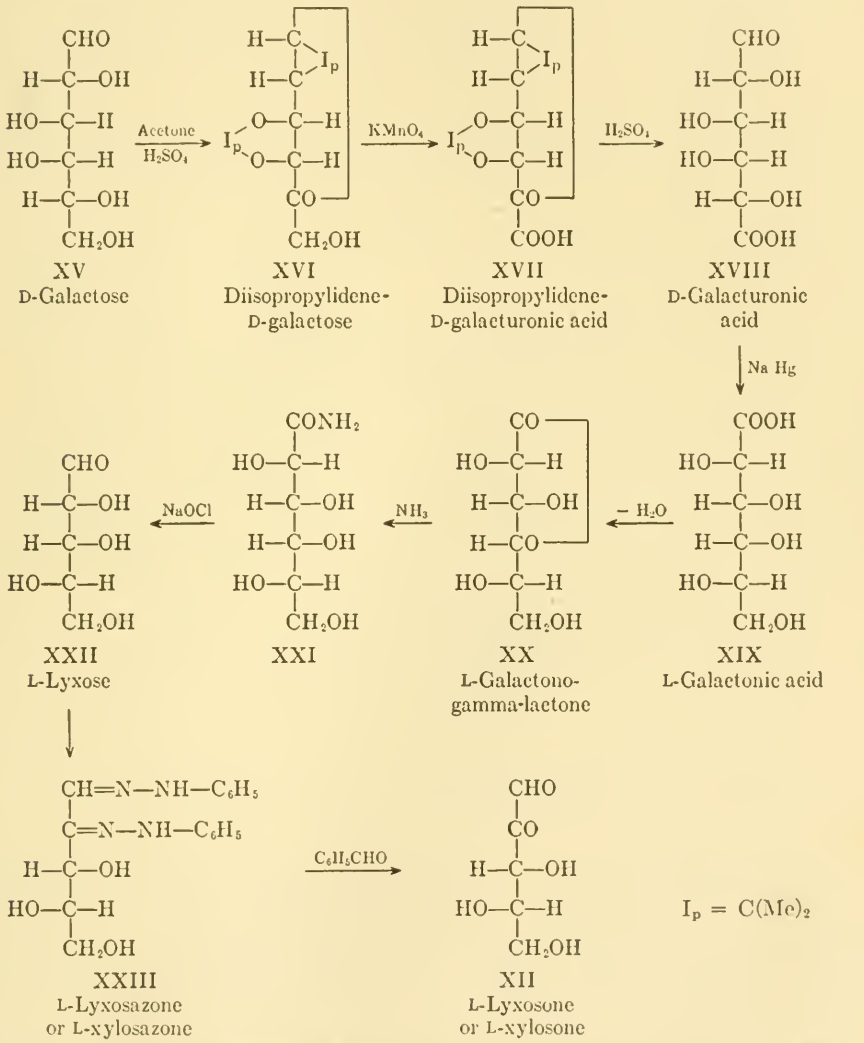
I — L-Ascorbic acid

This osone-cyanide method was employed almost simultaneously by two schools[19, 53-55] for the synthesis of L-ascorbic acid. The major problem in

[52] W. N. Haworth, E. L. Hirst, J. K. N. Jones, and F. Smith, *J. Chem. Soc.* **1934**, 1192.

this method involved the preparation of the somewhat inaccessible starting material L-xylosone or L-lyxosone (XII). Two pathways, involving reactions XV to XII (procedure a) and XXIV to XII (procedure b), devised for this purpose are formulated below.

Procedure a[54]

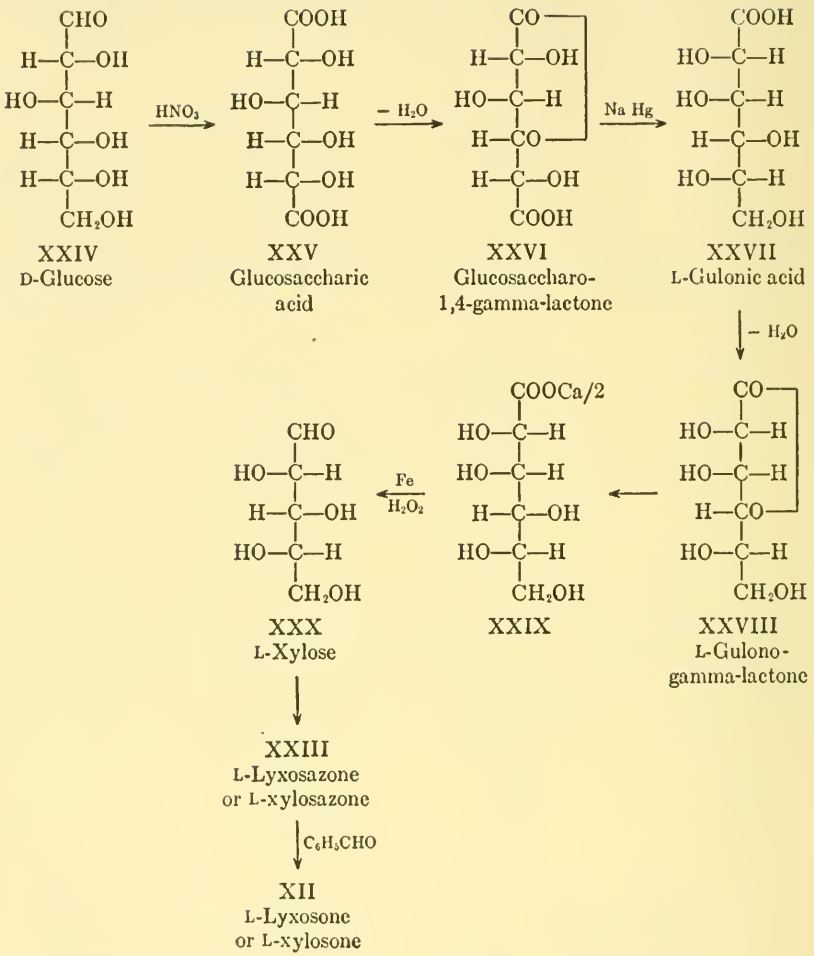


[53] T. Reichstein, A. Grüssner, and R. Oppenauer, *Helv. Chim. Acta* **16**, 561 (1938).

[54] R. G. Ault, D. K. Baird, H. C. Carrington, W. N. Haworth, R. W. Herbert, E. L. Hirst, E. G. V. Percival, F. Smith, and M. Stacey, *J. Chem. Soc.* **1933**, 1419.

[55] D. K. Baird, W. N. Haworth, R. W. Herbert, E. L. Hirst, F. Smith, and M. Stacey, *J. Chem. Soc.* **1934**, 62.

Procedure b[19]



The synthetic L-ascorbic acid obtained in this manner was chemically identical in every way with the naturally occurring vitamin C, and it was equally potent in curing scurvy in guinea pigs.[19, 20] This work marked an important step in organic chemistry, for L-ascorbic acid was the first vitamin to be synthesized.

The success of the osone method for synthesizing L-ascorbic acid and various analogs of ascorbic acid depends to no small extent on the purity of the sugar osazone. If the latter is a pentosazone, as was the case in the

synthesis of L-ascorbic acid, its conversion into the corresponding osone is best accomplished by means of benzaldehyde in hot aqueous alcohol,[54-56] whereas with the hexosazones, which are intermediates in the synthesis of the next higher members in the ascorbic acid series, it has been found advantageous to use fuming hydrochloric acid[52, 57] because, unlike the pentosazones and the methyl pentosazones, they are only sparingly soluble in aqueous alcohol and consequently they react very slowly with benzaldehyde. The oxidation of aldoses with cupric acetate in hot aqueous alcohol first effectively utilized by Weidenhagen[57a] has been reinvestigated,[57b, c] and it appears to be an excellent method for the preparation of osones. Although the osone-cyanohydrin reaction has been extensively used for the synthesis of L-ascorbic acid and its analogs[19, 46, 52-55, 58-61] on a small scale, it is not an economical method in spite of simpler alternative procedures for preparing osones,[57a, b, c, 62] and hence it is unsuitable for the large-scale manufacture of vitamin C.

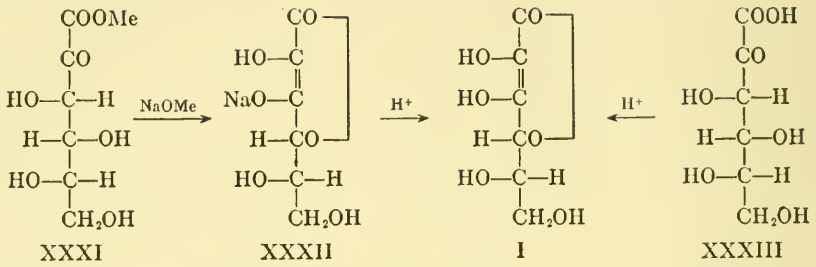
Method 2. Simultaneous Lactonization and Isomerization of an Appropriate 2-Keto Acid or Ester

This method is at present the best method for synthesizing vitamin C on a large scale, provided that the requisite keto acid or ester is available; it is also the most efficient for preparing analogs of ascorbic acid.

In the synthesis of L-ascorbic acid, methyl 2-keto-L-gulonate (XXXI), obtainable from the acid (XXXIII) by acid-catalyzed esterification,[63] by diazomethane,[63] and also by auto-esterification,[64] is boiled for a few minutes with a methanolic solution of sodium methoxide.[65, 66] The sodium ascorbate (XXXII) thus formed is then converted to the free ascorbic acid (I) by

[56] E. Fischer and E. F. Armstrong, *Ber.* **35**, 3141 (1902).
[57] E. Fischer, *Ber.* **22**, 87 (1889).
[57a] R. Weidenhagen, *Z. Wirtschaftsgruppe Zuckerind* **87**, 711 (1937).
[57b] L. L. Salomon, J. J. Burns, and C. G. King, *J. Am. Chem. Soc.* **74**, 5161 (1952).
[57c] J. K. Hamilton and F. Smith, *J. Am. Chem. Soc.* **74**, 5162 (1952).
[58] W. N. Haworth, E. L. Hirst, and J. K. N. Jones, *J. Chem. Soc.* **1937**, 549.
[59] Marguerite Steiger, *Helv. Chim. Acta* **18**, 1252 (1935).
[60] T. Reichstein, L. Schwarz, and A. Grüssner, *Helv. Chim. Acta* **18**, 353 (1935).
[61] T. Reichstein and V. Demole, *Festschr. Emil Barell* **107** (1936).
[62] I. Stone, U. S. Pat. 2,206,374 (1940).
[63] T. Reichstein and A. Grüssner, *Helv. Chim. Acta* **17**, 311 (1934).
[64] W. N. Haworth, E. L. Hirst, J. K. N. Jones, and F. Smith, British Pat. 443,901 (1936).
[65] K. Maurer and B. Schiedt, *Ber.* **66**, 1054 (1933); **67**, 1239 (1934).
[66] H. Ohle, H. Erlbach, and H. Carls, *Ber.* **67**, 324 (1934).

adding acid thus:



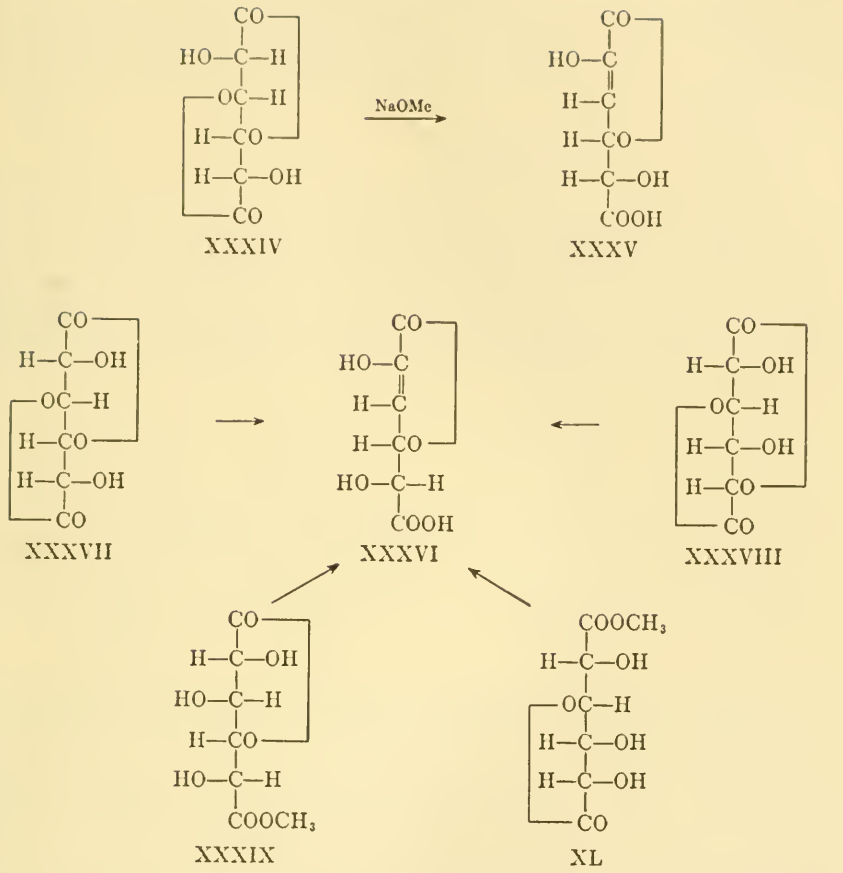
It is interesting to note that this reaction had been carried out on methyl 2-keto-D-gluconate[65] before L-ascorbic acid was synthesized, but the mechanism of the reaction was not known and thus the authors were unaware until later[55] that the glucosaccharosonic acid they had made was the first true analog of ascorbic acid, namely, D-araboascorbic acid.

A less convenient method for synthesizing L-ascorbic acid involving simultaneous dehydration and isomerization can be brought about by heating 2-keto-L-gulonic acid (XXXIII) alone[67, 68] or in the presence of an acid catalyst,[69] a reaction accelerated by carrying it out under pressure at an elevated temperature.[70]

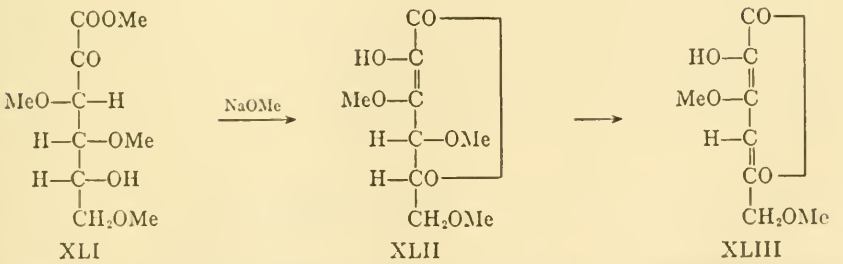
The type of isomerization leading to the formation of an unsaturated five-membered ring system by an internal type of Claisen condensation activated by carbonyl groups is more common than it appears. Thus, it is found in the alkaline conversion of mannosaccharodilactone (XXXIV) into the highly reducing substance 3-desoxy-6-carboxy-D-araboascorbic acid shown to have the formula XXXV[71] and in the formation of a related ascorbic acid-like substance, 3-desoxy-6-carboxy-L-ascorbic acid (XXXVI) from the dilactones (XXXVII), XXXVIII) and lactone esters (XXXIX, XL) of glucosaccharic acid.[72]

It is of interest to note that all these substances which undergo isomerization to give the ascorbic acid type of ring system possess two carbonyl groups as do those 2-keto esters which yield the true ascorbic acids. A further example of this interesting type of condensation and isomerization is seen in the case of XLI which upon treatment with sodium methoxide furnishes an analog of ascorbic acid which possesses a six-membered ring

[67] M. van Eckelen and P. J. van der Laan, Dutch Pat. 59,582 (1947).
[68] French Pat. 929,751 (1948).
[69] Belgian Pat. 452,811 (1943).
[70] Dutch Pat. 59,710 (1947).
[71] D. Heslop and F. Smith, *J. Chem. Soc.* **1944**, 577.
[72] D. Heslop and F. Smith, *J. Chem. Soc.* **1944**, 637.



and in which the unsaturation has proceeded one step further beyond the expected product, XLII to XLIII.[73]

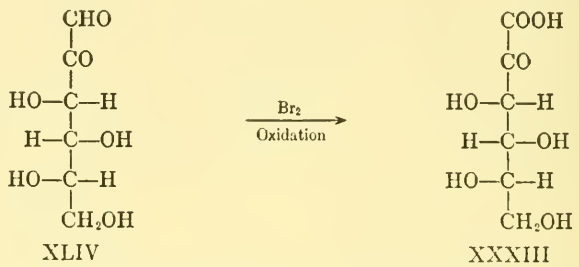


[73] W. N. Haworth, E. L. Hirst, and J. K. N. Jones, *J. Chem. Soc.* **1938**, 710.

The keto acids and their esters required for this particular method of synthesis have been made in a variety of ways which fall into four main groups.

1. Oxidation of osones.
2. Oxidation of aldonic lactones.
3. The conversion of an acid chloride to a keto nitrile followed by hydrolysis.
4. Oxidation of ketoses or certain of their carbonyl derivatives.

1. Oxidation of osones. When available, an osone such as L-gulosone (XLIV) can be oxidized with bromine to the corresponding 2-ketogulonic acid (XXXIII) in the manner previously established for the preparation of 2-keto-D-gluconic acid.[74]

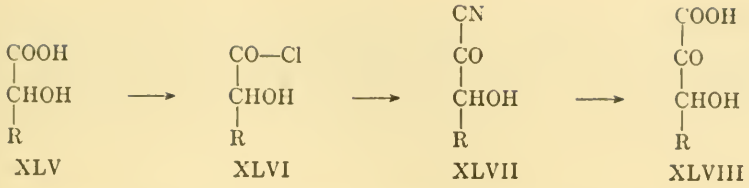


XLIV XXXIII

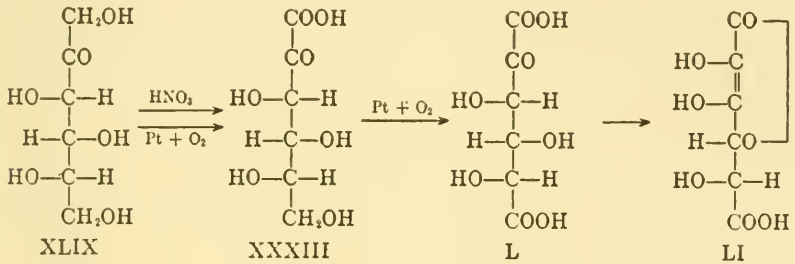
2. Oxidation of aldonic acids. An example of this apparently general reaction $\begin{matrix}\text{COOH}\\|\\\text{CHOH}\\|\end{matrix} \rightarrow \begin{matrix}\text{COOH}\\|\\\text{CO}\\|\end{matrix}$ is found in the oxidation of L-gulonic acid to 2-keto-L-gulonic acid with chromic acid[75] or with chromates in the presence of a vanadium catalyst.[76] This reaction, claimed to give good yields, has also been applied to D-[77] and to L-galactonic acid.[78]

3. The conversion of an acid chloride to a keto nitrile followed by hydrolysis. This method, which makes use of a classical reaction in organic chemistry, is also a general reaction and appears to be applicable to the preparation of L-ascorbic acid and its analogs. The sugar acid (XLV) is converted to the acid chloride (XLVI) which is then treated with silver cyanide to give the keto nitrile (XLVII). Hydrolysis of the latter with hydrochloric acid then provides the desired keto acid (XLVIII).[79]

[74] C. Neuberg and T. Kitasato, *Biochem. Z.* **183,** 485 (1927).
[75] R. Pasternak and P. P. Regna, U. S. Pat. 2,153,311 (1939).
[76] R. Pasternak and P. P. Regna, U. S. Pat. 2,188,777 (1940).
[77] P. P. Regna and B. P. Caldwell, *J. Am. Chem. Soc.* **66,** 243 (1944).
[78] H. S. Isbell, *J. Research Natl. Bur. Standards* **33,** 45 (1944).
[79] R. T. Major and E. W. Cook, U. S. Pat. 2,368,557 (1945).



4. Oxidation of ketoses or certain of their carbonyl compounds. Ketoses such as L-sorbose (XLIX) can be oxidized directly with nitric acid to the corresponding 2-keto acid. The early experiments were carried out by warming L-sorbose with dilute nitric acid,[64, 80] but more recently it has been recommended that the use of higher concentrations of nitric acid and a lower temperature[81] gives an increased yield of 2-keto-L-gulonic acid.

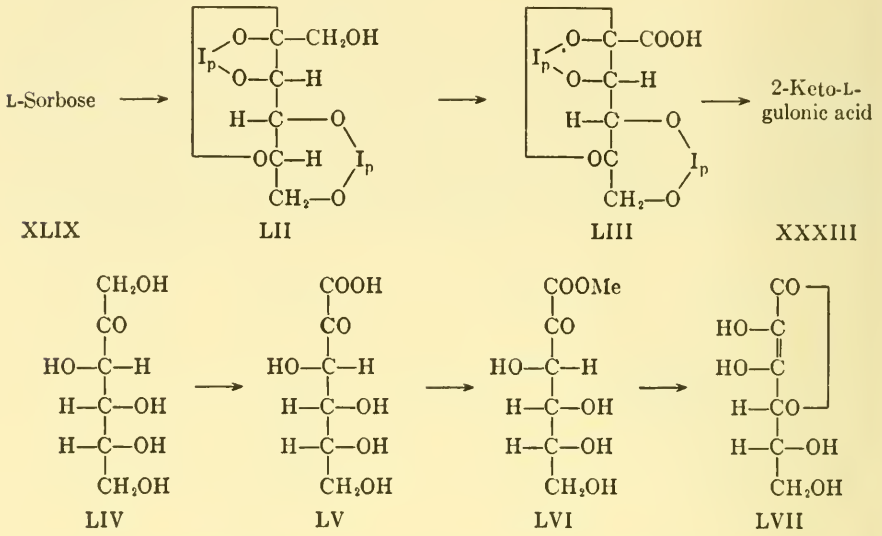


The direct oxidation of L-sorbose to 2-keto-L-gulonic acid has also been carried out in neutral[82] or slightly alkaline solution[83] by catalytic oxidation using a platinum catalyst. Conditions have also been so established that catalytic oxidation will proceed to 2-keto-L-gulosaccharic acid (L), and this in turn has been converted into L-gulosaccharoascorbic acid (6-carboxy-L-ascorbic acid, LI).[83] It is also reported that 2-keto-L-gulonic acid can be obtained from L-sorbose by electrolytic oxidation[84] and by oxidation with halooxygen compounds such as chlorites and chlorates.[85]

What appears to be the best and certainly the most convenient method of making 2-keto-L-gulonic acid (XXXIII) involves the conversion of L-sorbose (XLIX) to its 2,3-4,6-diisopropylidene derivative (LII). The latter, having a free primary alcoholic group, can be oxidized with permanganate in either acid[86] or alkaline solution[63] to yield 2,3-4,6-diiso-

[80] J. Overhoff and W. Huyser, U. S. Pat. 2,467,442 (1949).
[81] Dutch Pat. 59,301 (1947).
[82] N. R. Trenner, U. S. Pat. 2,483,251 (1949).
[83] K. Heyns, *Ann.* **558**, 177 (1947).
[84] Danish Pat. 68,836 (1949).
[85] S. Goldschmidt, Dutch Pat. 57,143 (1946).
[86] E. Boasson, S. Goldschmidt, and A. Middlebeek, Dutch Pat. 57,142 (1946).

propylidene-2-keto-L-gulonic acid (LIII). Upon autohydrolysis or treatment with acid, LIII gives rise to 2-keto-L-gulonic acid (XXXIII). In forming the acetone derivative (LII), there appears to be some advantage in doing the reaction at lower temperatures than those normally used.[87]



A similar series of reactions has been applied in the conversion of D-fructose (LIV) to 2-keto-D-gluconic acid (LV) which is readily transformed into its methyl ester (LVI) and then into D-araboascorbic acid (LVII).[65, 66, 88]

Numerous references are to be found in the patent literature relating to methods of making L-ascorbic acid which are fundamentally related to those mentioned above. Mention may be made here, perhaps, of the procedure whereby the 2,3-4,6-diisopropylidene-2-ketogulonic acid is converted directly into L-ascorbic acid by treatment with an acid catalyst, for example hydrogen chloride, in a solvent or mixture of solvents in which the diisopropylidene compound is soluble but in which L-ascorbic acid is insoluble. Such conditions result in the crystallization of L-ascorbic acid from the reaction mixture.[89-92] In another method, molten diiospropylidene-

[87] I. T. Strukov and N. A. Kapylova, *Farmatsiya* **10**, No. 3, 8 (1947) [*C.A.* **44**, 8327 (1950)].

[88] D. Heslop, E. Salt, and F. Smith, *J. Chem. Soc.* **1944**, 225.

[89] T. Reichstein, British Pat. 446,548 (1937).

[90] R. Pasternak and G. O. Cragwell, U. S. Pat. 2,184,383 (1940).

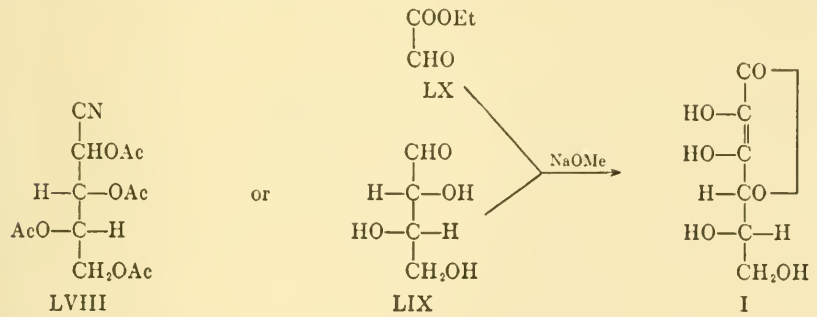
[91] V. M. Berezovskiĭ and L. I. Strel'chunas, *Zhur. Priklad. Khim.* **22**, 1113 (1949) [*C.A.* **45**, 5627 (1951)].

[92] H. W. Bassford, Jr., W. S. Harmon, and J. F. Mahoney, U. S. Pat. 2,462,251 (1949).

2-keto-L-gulonic acid at 120° is treated with a halogen acid. Within a few minutes the conversion is complete and a 70 to 80% yield of L-ascorbic acid can be isolated.[70] Finally, it may be noted that 2-keto esters such as XXXI and LVI can be converted into the corresponding ascorbic acid by heating in aqueous solution with magnesium, iron, nickel, cobalt, cadmium, and zinc.[93, 94] As might have been anticipated, other workers have used esters other than the methyl, namely, the butyl and ethyl esters.[95, 96]

Method 3. Benzoin Condensation of Sugars with Ethyl Glyoxylate or Ethyl Mesoxalate

This excellent method of general application may be used not only for the synthesis of ascorbic acid but for its many analogs. Thus, L-threose (LIX) or tetraacetyl-L-threose cyanohydrin (LVIII), which affords L-threose upon treatment with alkali, will condense with ethyl glyoxylate (LX) in an alkaline medium to give L-ascorbic acid (I).[97] Ethyl mesoxalate[98] can



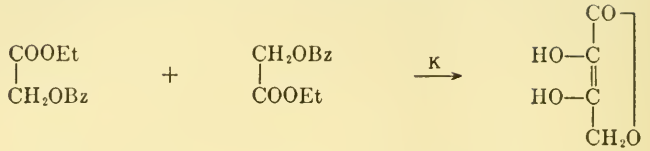
replace ethyl glyoxylate in this reaction. The method is limited in some instances by the inaccessibility of some of the starting materials, as is the case with L-threose. Recently it has been claimed that ethyl hydroxy ethoxyacetate is superior to ethyl glyoxylate for use in the above condensation, 90% yields of vitamin C being reported.[99]

Method 4. Claisen Condensation of Esters of Hydroxy Acids

The simplest true analog of ascorbic acid, oxytetronic acid (LXII), is derived from the action of potassium upon ethyl benzoyl oxyacetate[100]

[93] R. Pasternak and P. P. Regna, U. S. Pats. 2,165,151; 2,165,184 (1939).
[94] Japanese Pat. 161,770 (1944).
[95] A. H. Ruys and J. F. Lemmens, U. S. Pat. 2,491,933 (1950).
[96] British Pat. 601,789 (1948).
[97] B. Helferich and O. Peters, *Ber.* **70**, 465 (1937); German Pat. 637,448 (1936).
[98] B. Helferich, German Pat. 683,954 (1939); U. S. Pat. 2,207,680 (1940).
[99] P. L. Stedehouder, Dutch Pat. 59,711 (1947).
[100] F. Micheel and F. Jung, *Ber.* **66**, 1291 (1933).

(LXI) by a Claisen type of condensation thus:



LXI LXII

Attempts have been made to extend this reaction to the synthesis of hydroxy tetronic acid having the ascorbic acid side chain.[101]

E. STRUCTURE AND ANTISCORBUTIC ACTIVITY OF ANALOGS OF L-ASCORBIC ACID

The methods outlined above have enabled a number of analogs of L-ascorbic acid to be made. Although their structures have not all been determined, the work on two of them, namely D-glucoascorbic acid[58] and D-araboascorbic acid,[58, 102] strongly suggests that all have the same dienolic five-atom ring found in L-ascorbic acid.

The antiscorbutic activity of these analogs has been compared with that of L-ascorbic acid (see Tables I, II, and III below).

Inspection of these formulas reveals the interesting fact that for antiscorbutic activity (see Table I) the five-atom lactone ring must lie to the right of the carbon chain when the formula is written according to the Fischer convention. Apparently no exception to this rule has been brought to light. Methyl 2-keto-L-gulonate has been reported to have antiscorbutic activity,[61] but presumably this is due to the fact that it undergoes conversion into L-ascorbic acid in the animal. It has also been observed that, although dehydroascorbic acid or 2,3-diketo-L-gulono-γ-lactone (II) is fully

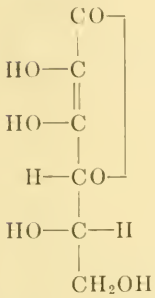
[101] F. Micheel and H. Haarkoff, *Ann.* **545**, 28 (1940).
[102] E. G. E. Hawkins, E. L. Hirst, and J. K. N. Jones, *J. Chem. Soc.* **1939**, 246.
[103] H. Müller and T. Reichstein, *Helv. Chim. Acta* **21**, 273 (1938).
[104] V. Demole, *Biochem. J.* **28**, 770 (1934).
[105] O. Dalmer and T. Moll, *Hoppe-Seyler's Z. physiol. Chem.* **222**, 116 (1933).
[106] S. S. Zilva, *Biochem. J.* **29**, 1612 (1935).
[107] T. Reichstein, *Helv. Chim. Acta* **17**, 1003 (1934).
[108] T. Reichstein and R. Oppenauer, *Helv. Chim. Acta* **16**, 988 (1933).
[109] H. von Euler and C. Martius, *Svensk Kem. Tidskr.* **45**, 73 (1933).
[110] H. von Euler, H. Hasselquist and U. Lööv, *Arkiv. Kemi, Mineral. Geol.* **26B**, No. 5, 7 (1948).
[111] W. Cocker, R. A. Q. O'Meara, J. C. P. Schwarz, and E. R. Stuart, *J. Chem. Soc.* **1950**, 2052.
[112] F. Micheel and R. Mittag, *Hoppe-Seyler's Z. physiol. Chem.* **247**, 34 (1937).
[113] F. Micheel, G. Bode, and R. Siebert, *Ber.* **70**, 1862 (1937).
[114] J. R. Penney and S. S. Zilva, *Biochem. J.* **37**, 403 (1943).

as active as L-ascorbic acid, the open-chain 2,3-diketo-L-gulonic acid (III) is inactive.[114] It is also very likely that the unsaturated five-membered ring must have both enolic hydroxyl groups, since the closely related 3-desoxy-L-gulosaccharoascorbic acid[72] mentioned previously is inactive;[115] the syn-

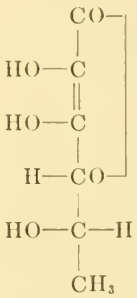
TABLE I

ANTISCORBUTICALLY ACTIVE SUBSTANCES

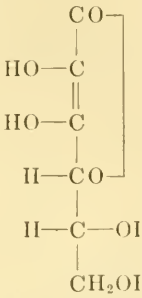
(The fraction in parentheses indicates the antiscorbutic activity on the basis of unit activity for L-ascorbic acid.)



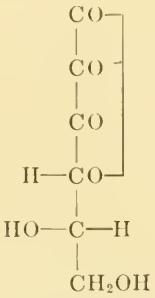
L-Ascorbic acid (vitamin C)[19, 20, 33, 54, 104]
m.p. 192°, $[\alpha]_D$ + 23°
(1)



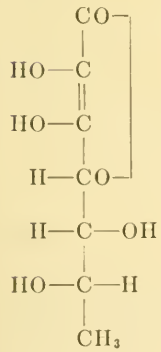
6-Desoxy-L-ascorbic acid [103]
m.p. 168°, $[\alpha]_D$ + 37°
(1/3)



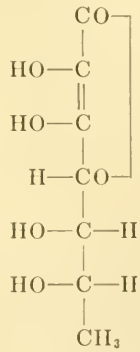
D-Araboascorbic acid[46, 65, 66, 104-106]
m.p. 174°, $[\alpha]_D$ − 17°
(1/20)



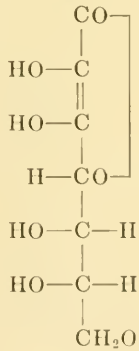
Dehydro-L-ascorbic acid [32, 33, 127]
m.p. 225°, $[\alpha]_D$ + 55°
(1)



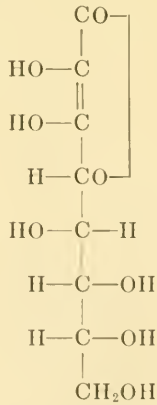
L-Fucoascorbic acid[61]
—
(1/50)



L-Rhamnoascorbic acid[60, 106]
m.p. 199°, $[\alpha]_D$ + 28°
(1/5)



L-Glucoascorbic acid[58, 61, 104, 106]
m.p. 140°, $[\alpha]_D$ + 24°
(1/40)



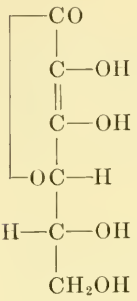
D-Glucoheptoascorbic acid[61, 97]
—
(1/100)

[116] D. Heslop and F. Smith, Unpublished results.

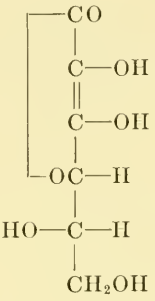
thetic 2-amino[112] and 2,3-diamino[113] derivatives of L-ascorbic acid are likewise inactive. Reference may also be made to reductinic acid, produced from glucuronic acid, pectin, and furfural,[108] and to reductone which is

TABLE II

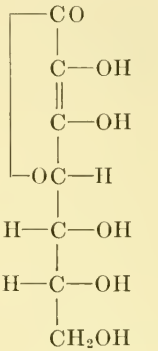
ANTISCORBUTICALLY INACTIVE SUBSTANCES



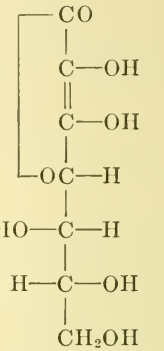
D-Xyloascorbic acid (D-ascorbic acid)[19, 54, 104, 106] m.p. 192° $[\alpha]_D$ − 23°



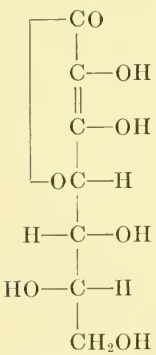
L-Araboascorbic acid[46, 55, 104] m.p. 174°, $[\alpha]_D$ − 17°



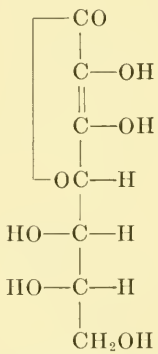
D-Glucoascorbic acid[46, 54, 104, 106] m.p. 140° (monohydrate), $[\alpha]_D$ − 22°



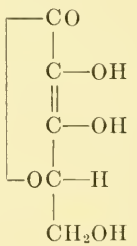
D-Galactoascorbic acid[46, 55, 104, 106] m.p. 134°, $[\alpha]_D$ − 6°



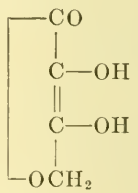
L-Guloscorbic acid [46, 61, 104] m.p. 184°, $[\alpha]_D$ − 22°



L-Alloascorbic acid [59] m.p. 177°, $[\alpha]_D$ + 29°



L-Erythroascorbic acid[107] m.p. 161°, $[\alpha]_D$ + 9°



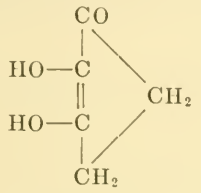
Hydroxytetronic acid[100, 105] m.p. 153°, optically inactive

formed from sugars such as glucose by treatment with alkalies[109-111] (see Table III). Both show the same vigorous reducing properties and similar light absorption characteristics as L-ascorbic acid, but both are inactive. Dihydroxymaleic acid and hydroxytetronic acid, referred to above (Table III), are also inactive.

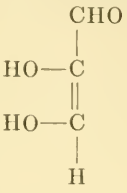
The fundamental knowledge of the biochemical function of L-ascorbic acid is still rather limited, and although its sole function seems to be one of preventing scurvy, it is not unlikely that this disease is the manifestation of a number of biochemical processes that have got out of order. If and when these fundamental processes are brought to light, it will be of interest, perhaps, to reinvestigate some of the above analogs of L-ascorbic acid to see whether L-ascorbic acid does indeed have polyfunctional biological properties. Quite recently May and his associates have been investi-

TABLE III

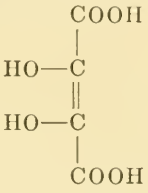
ANTISCORBUTICALLY INACTIVE SUBSTANCES RELATED TO ASCORBIC ACID



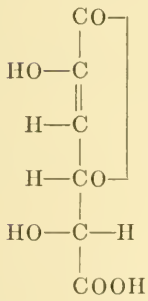
Reductinic acid[108]



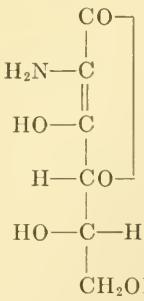
Reductone[109-111]



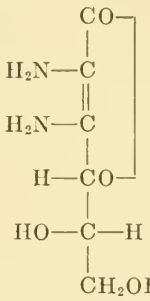
Dihydroxymaleic acid



3-Desoxy-L-gulosaccharoacorbic acid[72, 115]



2-Amino-L-ascorbic acid[112]



2,3-Diamino-L-ascorbic acid[113]

gating the effect of D-ascorbic acid and various analogs of L-ascorbic acid on scorbutic monkeys showing symptoms of megaloblastic anemia, and it seems that L-ascorbic acid (vitamin C) may indeed be polyfunctional in its behavior.[115a] Samples of L-ascorbic acid labeled at C_1[116, 57b, c] with radioactive C^{14} and labeled "uniformly"[117] have been made by methods 1 and 2 discussed above (see pp. 188 and 191), and it is very likely that a study of these materials in biological systems will throw light on the fate and function of ascorbic acid.[118]

[115a] C. D. May, A. Hamilton, C. T. Stewart, *Blood* **7**, 978 (1952).
[116] J. J. Burns and C. G. King, *Science* **111**, 257 (1950).
[117] A. A. Bothner-By, M. Gibbs, and R. C. Anderson, *Science* **112**, 363 (1950).
[118] J. J. Burns, H. B. Burch, and C. G. King, *J. Biol. Chem.* **151**, 501 (1951).

In the case of rats, for which L-ascorbic acid is not a vitamin, it appears that when they are stimulated into producing excessive amounts of L-ascorbic acid, by administration of chloretone, they do so at the expense of glucose; this has been established by feeding the rats D-glucose-1-C^{14} and showing that the excreted L-ascorbic acid contains radioactive carbon mainly at C_6.[118a]

F. DERIVATIVES OF L-ASCORBIC ACID

The physiological activity and the novel dieneolic system of L-ascorbic acid have led to much experimentation by workers in the fields of pure chemistry and the applied biological sciences. From the various derivatives referred to below it will be apparent that the hydroxyl groups located at C_5 and C_6 behave in the normal manner whereas the other two hydroxyl groups located at C_2 and C_3, the CO group of the five-membered lactone ring, and the double bond display what at one time were thought to be very unexpected properties. Thus, the acidity is due not to the lactone ring but to the hydroxyl groups at C_2 and C_3, the one at C_3 being much more acidic (pK 4.1) than that at C_2 (pK 11.6),[119] a deduction previously made from the methylation experiments with diazomethane.[46, 47] Hence, in the presence of alkali the lactone ring does not open but instead a proton is expelled from the OH at C_3; this behavior, with the subsequent transformation of the ionized ring system into a state of resonance, no doubt explains the high rotation of the sodium salt and the movement of the absorption band towards longer wavelengths.[52] Similarly, it is found that the double bond is also rather unique inasmuch as reagents such as chlorine and bromine, which normally add to a double bond, bring about saturation of the bond by oxidation to dehydroascorbic acid. It is also interesting to note that, after the acidity of the hydroxy groups at C_2 and C_3 has been neutralized by methylation with diazomethane to give the dimethyl ether (IX) of ascorbic acid, the lactone ring can be opened but then only by the concomitant saturation of the double bond by ring closure between C_6 and C_3 (see later).

1. Dehydroascorbic Acid

From the biochemical point of view, this is perhaps one of the more important derivatives of L-ascorbic acid because it still retains the same antiscorbutic activity as the vitamin itself.[33] Although easily produced in solution, it is also sensitive to oxidation and until relatively recently it was not possible to isolate it in good yield in the crystalline state.[120] It may be

[118a] H. H. Horowitz, A. P. Doerschuk, and C. G. King, *J. Biol. Chem.* **199**, 193 (1952).
[119] T. W. Birch and L. J. Harris, *Biochem. J.* **27**, 595 (1933).
[120] J. Kenyon and N. Munro, *J. Chem. Soc.* **1948**, 158.

produced in solution by the action of the halogens; chlorine, bromine, and iodine, acid potassium permanganate,[34] ferric salts, [37] quinone, phenolindophenol,[8] 2,6-dichlorophenolindophenol,[35] methylene blue,[36] and perinaphthindane trione.[121] The formation of dehydroascorbic acid is a well-defined reaction and, consequently, a number of the above reagents have been found to give excellent results in determining L-ascorbic acid in solutions of the pure substance or in the absence of interfering substances (see also p. 253). Many attempts have been made to apply such methods, including potentiometric[122] and polarographic[123] and colorimetric[124] procedures, to the determination of the amount of vitamin C in various plant and animal tissues and various biological specimens, but as far as this author is aware there seems to be no single method which is generally applicable.[38] Interference from a number of natural products makes the determination of L-ascorbic acid in any one type of natural product a major research in itself.[125, 126] If stabilization of the L-ascorbic acid in an extract can be ensured, it would appear that paper partition chromatography might be useful in dealing with this problem.

Freshly prepared samples of dehydroascorbic acid (II), unlike solutions of the parent vitamin, show no selective absorption in the ultraviolet region of the spectrum and, consequently, it is believed to exist in the hydrated form (III);[32] in alcohol it appears to form an alcoholate and, indeed, when the dehydro compound is properly prepared it crystallizes readily from methanol with solvent of crystallization.[127] Dehydroascorbic acid undergoes hydrolysis in aqueous solution,[32, 120] and in the presence of air[128] and traces of copper[39] it readily decomposes to give oxalic acid. In alkaline solution in the presence or absence of air it undergoes profound degradation. It is thus apparent that especial care must be taken in extracting natural products and preserving the extracts, in any problems which have to do with isolation or analysis. Acids, and preferably those such as *m*-phosphoric

[121] M. S. El Ridi, R. Moubasher, and Z. F. Hassan, *Biochem. J.* **49**, 246 (1951).

[122] L. J. Harris, L. W. Mapson, and Y. L. Wang, *Biochem. J.* **36**, 183 (1942); C. Spacu and P. Spacu, *Z. anal. Chem.* **128**, 233 (1948).

[123] R. Perrin and D. D. Perrin, *New Zealand J. Sci. Technol.* **28A**, 266 (1946); K. Weisner and K. Schaferna, *Chem. Listy* **38**, 211 (1944) [*C.A.* **44**, 5948 (1950)]; D. M. Coulson, W. R. Crowell, and S. L. Friess, *Anal. Chem.* **22**, 525 (1949).

[124] W. B. Robinson and E. Stotz, *J. Biol. Chem.* **160**, 217 (1945); G. Mannelli, *Mikrochemie ver. Mikrochim. Acta* **35**, 29 (1950); B. Naganna and P. Ramachandra Rao, *Current Sci. (India)* **18**, 250 (1949).

[125] G. W. Probst and M. O. Schultze, *J. Biol. Chem.* **187**, 453 (1950).

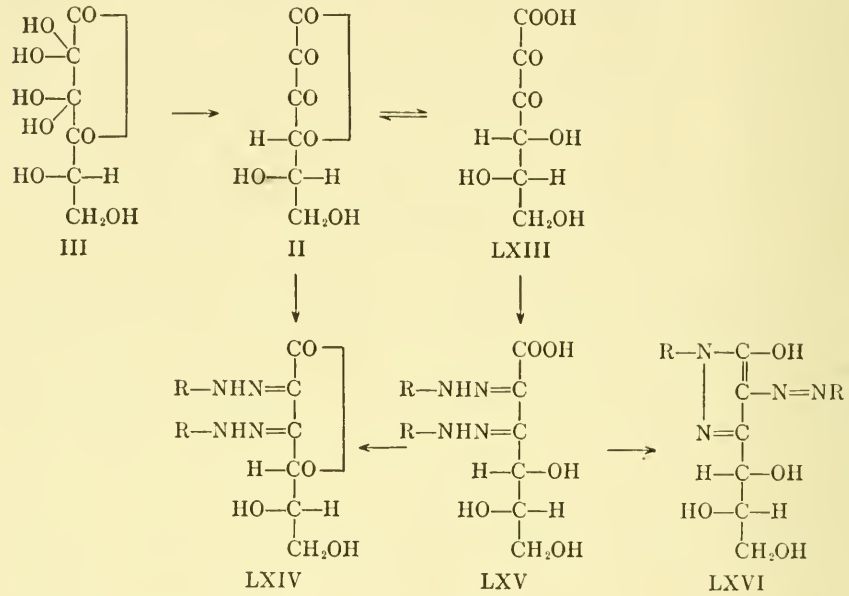
[126] S. S. Jackel, E. H. Mosbach, and C. G. King, *Arch. Biochem. and Biophys.* **31**, 442 (1951).

[127] B. Pecherer, *J. Am. Chem. Soc.* **73**, 3827 (1951).

[128] B. Rosenfeld, *J. Biol. Chem.* **150**, 281 (1943).

acid[129] and oxalic acid[130] which complex copper ions, are therefore recommended for the extraction which should be done as expeditiously as possible at room temperature to reduce losses by oxidation to a minimum. Solutions of L-ascorbic acid are stabilized to some extent by sodium chloride,[131] borates,[132] and various inorganic and organic compounds of sulfur.[133]

Since dehydroascorbic acid (II) is the lactone of a 2,3-diketoaldonic acid (LXIII), with which it is in equilibrium in aqueous solution, it is not



R = C_6H_5—, *p*-Br·C_6H_4—, *p*-NO_2·C_6H_4—, and 2,4-$(NO_2)_2$·C_6H_3—

surprising to find that it readily combines with phenylhydrazine and its derivatives. Osazones corresponding to both the open and closed chain forms of dehydroascorbic acid can readily be obtained. The osazone (LXV) of the diketo acid is easily converted into the osazone (LXIV) of the diketo lactone simply by recrystallization. A third compound obtained during the preparation of these osazones is the pyrazolone (LXVI) which can be obtained from (LXV) by treatment with alkali.[49] These compounds can be

[129] P. György, *In* Vitamin Methods, p. 260. Academic Press, New York, 1950; Methods of Vitamin Assay, 2nd ed., p. 71. Interscience Publishers, New York, 1951.
[130] J. D. Ponting, *Ind. Eng. Chem., Anal. Ed.* **15**, 389 (1943); F. Wokes, *Analyst* **72**, 63 (1947); F. E. Hailin and I. M. Stephens, *Australian J. Exptl. Biol. Med. Sci.* **25**, 17 (1947); L. P. Guild, E. F. Lockhart, and R. S. Harris, *Science* **107**, 226 (1948).
[131] T. Marx, *Landwirtsch Forsch.* **2**, 229 (1951).
[132] W. E. Militzer, *J. Biol. Chem.* **158**, 247 (1945).
[133] R. Stadelmann, *Schweiz. Apoth. Ztg.* **85**, 865 (1947).

obtained directly from L-ascorbic acid,[32] but the reaction is slow and is accompanied by some decomposition since oxidation precedes osazone formation. The reaction between dehydroascorbic acid and 2,4-dinitrophenylhydrazine which gives a product corresponding to LXIV in good yield forms the basis of one of the better analytical methods for determining the ascorbic acid content of plant and animal products.[38, 125, 126, 134] Similar derivatives have been obtained with *o*-phenylenediamine[48] and *p*-sulfamylphenylhydrazine.[135] It has also been shown that, in its reactions with the various phenylhydrazines[32] and *o*-phenylenediamine, L-ascorbic acid is paralleled by the behavior of D-araboascorbic acid.[48, 49]

2. Acyl and Aryl Derivatives

L-Ascorbic acid does not lend itself easily to acetylation, although it is claimed that the 5,6-diacetate can be obtained without too much difficulty.[41] An oily tetraacetate has been prepared by vigorous acetylation.[40] Of more interest from a structural point of view is the observation that treatment of 5,6-monoacetone L-ascorbic acid with ketene, until it gives no color reaction with phenolindophenol, yields the 3-acetyl-5,6-monoacetone derivative, thus showing the more active nature of the OH at C_3,[136] while a suspension of L-ascorbic acid in acetone treated with ketene affords 2,3-diacetyl-5,6-monoacetone L-ascorbic acid.[137] Various 2,3-diphenacyl ethers and their corresponding 5,6-diacetates have been prepared in an attempt to determine the activity of ascorbic acid when its dienol system is blocked.[138]

3. Ethers

The methyl ethers were of primary importance because it was the study of the 2,3,5,6-tetramethyl ether of ascorbic acid which finally led to the proof of the structure of L-ascorbic acid. These methyl ethers have also revealed in a striking manner the unique chemical nature of the vitamin molecule.

The hydroxyl group at C_3 of the ascorbic acid molecule (I) is so much more acidic than that at C_2 that it can be preferentially titrated with diazomethane to give 3-methyl-L-ascorbic acid (LXVIII), a crystalline substance that gives a blue color with ferric chloride.[44, 46, 47] Simultane-

[134] J. H. Roe and C. A. Kuether, *J. Biol. Chem.* **147**, 399 (1943); J. H. Roe and M. J. Osterling, *ibid.* **152**, 511 (1944); O. A. Bessey, O. H. Lowry, and M. J. Brock, *ibid.* **168**, 197 (1947).

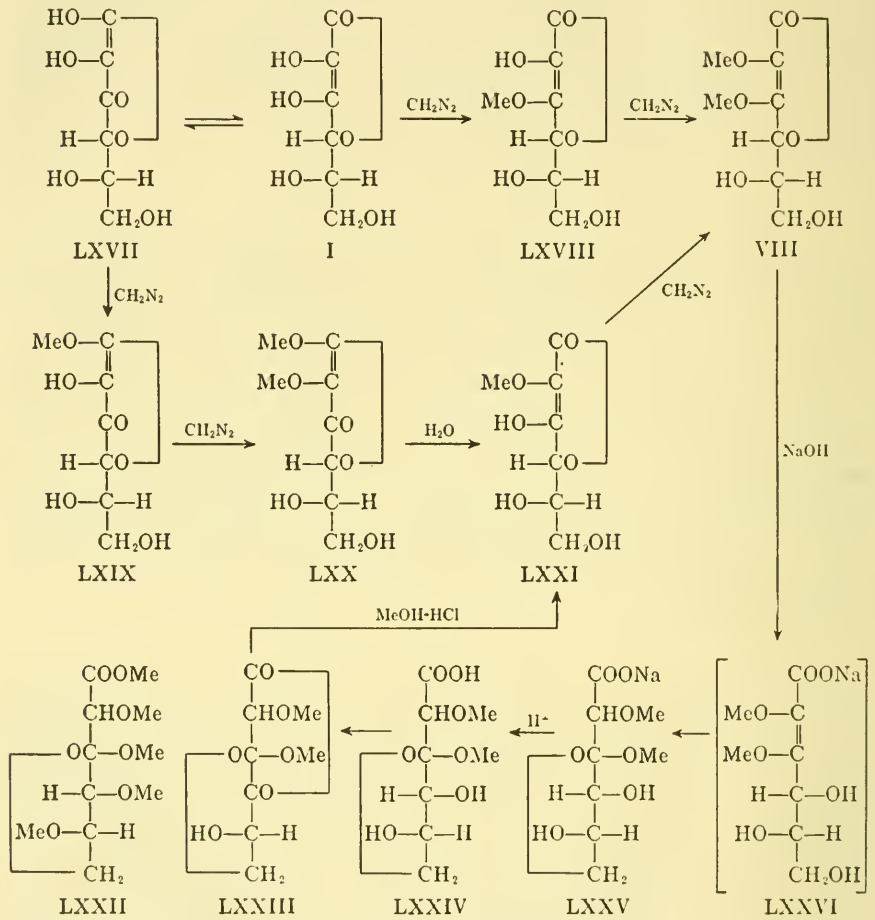
[135] H. Willstaedt, *Svensk. Kem. Tidskr.* **55**, 214 (1943).

[136] C. S. Vestling and M. C. Rebstock, *J. Biol. Chem.* **152**, 585 (1944).

[137] Y. Sumiki, S. Yamanaka, K. Oka, and A. Takeishi, *J. Agr. Chem. Soc. Japan* **20**, 89 (1944).

[138] C. S. Vestling, and M. C. Rebstock, *J. Biol. Chem.* **161**, 285 (1945).

ously, there is formed an isomeric monomethyl ether 1-methyl heteroascorbic acid (LXIX) characterized by the red color it gives with ferric chloride.[44, 47] The identification of these two monomethyl ethers shows the facility with which ascorbic acid can pass into its tautomeric modifications.



The monomethyl heteroascorbic acid (LXIX) has been shown to possess high antiscorbutic activity,[139] no doubt because it readily reverts to L-ascorbic acid. There seems to be some difference of opinion as to how active the 3-methyl-L-ascorbic acid is,[139, 140] but according to the most recent work it probably has about one-fiftieth the activity of L-ascorbic acid.[139] The

[139] B. S. Gould, H. M. Goldman, and J. T. Clarke, *Arch. Biochem.* **23**, 205 (1949); H. M. Goldman and B. S. Gould, *J. Nutrition* **43**, 193 (1951).

[140] C. S. Vestling and M. C. Rebstock, *J. Biol. Chem.* **164**, 631 (1946).

TABLE IV

DERIVATIVES OF L-ASCORBIC ACID (VITAMIN C)

Derivative	M. p., °C.	$[\alpha]_D$ (solvent)	Reference
3-Acetyl-5,6-isopropylidene	116	+27° (MeOH)	136
2-Amino- (scorbamic acid)	100	—	112
6-Carboxy- (L-gulosaccharoascorbic acid)	206–210	—	82
Dehydro-[a]	220–225	+55° (H_2O)	120, 127
2-Desoxy-	170	—	142
6-Desoxy-	168	+37° (H_2O)	103
5,6-Diacetyl-	157	+64° (H_2O)	41
2,3-Diacetyl-5,6-isopropylidene	116		137
1,2-Dimethyl-	Liquid	+107° (MeOH)	44
2,3-Dimethyl-	59 (monohydrate)	+32° (MeOH)	43, 47
5,6-di-*p*-nitrobenzoyl-	172	−79° ($CHCl_3$)	43
6-trityl-	156, 178 (dimorphous)	+35° ($CHCl_3$)	44, 141
5,6-isopropylidene	101	+16° (EtOH)	43
2,3- Dimethyl iso-	Liquid	−18° (MeOH)	44, 143
1-amide-	120	−23° (MeOH and H_2O)	32, 43, 44
5-*p*-nitrobenzoyl-	180	+19° ($CHCl_3$)	44, 143
5,6-Isopropylidene	222 d.	+20° (H_2O) +28° (MeOH)	144
1-Methyl hetero-	162	+240° (MeOH)	44, 46, 47
2-Methyl-	Liquid	+10° (H_2O)	44
3-Methyl-	121	+29° (H_2O)	44, 46, 47
5,6-isopropylidene	88–90	+20° (MeOH)	46
1,2,3,4,5-Pentamethyliso-	Liquid	−51° (MeOH)	47
2,3,5,6-Tetramethyl-	Liquid	+11° ($CHCl_3$)	32, 47
2,3,5-Trimethyl-	69–70	−11° (H_2O)	44
6-*p*-nitrobenzoyl-	118	±0° ($CHCl_3$)	44
6-trityl-	131	+32° ($CHCl_3$)	44
2,3,5-Trimethyliso-	38	−35° (H_2O)	44
1-amide-	115	−35° (MeOH)	44
Compound with			
Benzophenone	208		145
Nicotinic acid	185	+11° (H_2O)	146
Nicotinamide	145	+27° (H_2O)	146

[a] For derivatives of dehydroascorbic acid with phenylhydrazine, substituted phenylhydrazines, and *o*-phenylenediamine, see refs. 32, 49, and 135.

[141] F. Micheel and G. Bischoff, *Ann.* **525**, 66 (1936).

[142] F. Micheel and K. Hasse, *Ber.* **69**, 879 (1936).

[143] F. Micheel and W. Schulte, *Ann.* **519**, 70 (1935).

[144] L. von Vargha, *Nature* **130**, 846 (1932); **131**, 363 (1933).

[145] P. E. Papadakis, *J. Am. Chem. Soc.* **67**, 2051 (1945).

[146] W. Wenner, *J. Org. Chem.* **14**, 22 (1949).

1-methyl hetero ether (LXIX) shows an absorption band at a much longer wavelength than the normal 3-methyl ether (LXVIII), and it also has a larger specific rotation. Upon further methylation of the 3-methyl ether it gives the normal 2,3-dimethyl ether (VIII), whereas the 1-methyl ether (LXIX) yields the corresponding 1,2-dimethyl heteroascorbic acid (LXX). In aqueous solution VIII is stable, but the 1,2-dimethyl hetero compound (LXX) loses the methyl group from C_1 to give a 2-methyl ether (LXXI) which must have the normal ascorbic acid structure, since upon methylation it gives rise to 2,3-dimethyl-L-ascorbic acid (VIII).[44] The latter crystallizes as a monohydrate, but the melting point is rather low and it is advisable to characterize it as the 5,6-monoisopropylidene derivative[43] or the 5,6-di-*p*-nitrobenzoate[43] or as the 6-trityl ether which occurs in two crystalline modifications.[44, 141]

Another curious type of isomerization is displayed by 2,3-dimethyl-L-ascorbic acid (VIII) when it is allowed to react at room temperature with dilute alkalies in which treatment one equivalent of base is consumed. By analogy with simple lactones LXXVI would be expected, but evidently such a system, having its electrons displaced from the double bond by the ionized carboxyl group, is unstable, and stabilization is attained by saturation of the double bond through closure of the ring between C_3 and C_6 to give a salt (LXXV) of the methyl furanoside of a 3-keto acid, a compound which shows no selective absorption in the ultraviolet. Upon acidification of LXXV it affords the free acid furanoside (LXXIV), which readily undergoes further ring closure to give the furanoside γ-lactone (LXIII). Treatment of the latter with ammonia readily gives, by opening of the 1,4-lactone ring, a crystalline amide which may also be obtained in smaller yield directly from the normal 2,3-dimethyl-L-ascorbic acid (VIII). The cycle of isomerizations may now be completed, for by boiling LXXIII with methyl alcoholic hydrogen chloride the furanoside methyl group is eliminated from C_3 with the formation of the 2-methyl-L-ascorbic acid. Further structural information is derived from the fact that methylation of VIII with methyl sulfate followed by esterification was found to give a pentamethyl compound (LXXII).[44]

The type of isomerization which takes place when 2,3-dimethyl-L-ascorbic acid is transformed into its iso compound by the agency of alkalies has also been shown to occur with the 2,3,5-trimethyl- but not with the 2,3,5,6-tetramethyl-L-ascorbic acid, thus showing that the $-CH_2OH$ group at C_6 is involved in the isomerization.[44]

The transformations and isomerizations discussed above in connection with 2,3-dimethyl-L-ascorbic acid are evidently general reactions inasmuch as 2,3-dimethyl-D-glucoascorbic acid behaves in an analogous manner.[58]

III. Industrial Preparation

FRED SMITH

The "enediol" ring system present in ascorbic acid may be produced by a number of methods (see p. 188) and the patent literature shows that most of them have been investigated with the object of making L-ascorbic acid on a large scale.

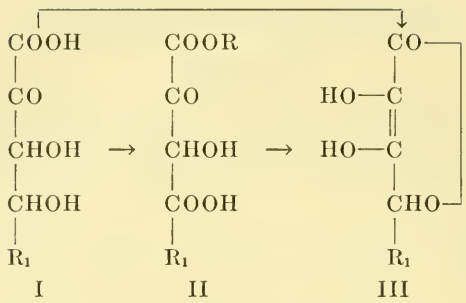
The osone method requires L-lyxosone (L-xylosone) which may be made from either L-lyxose[1] or L-xylose.[2] However, in spite of the fact that osones can be prepared in good yield directly from the sugars with cupric acetate by two methods, one made possible by the use of methyl alcohol as a solvent[3-5] and the other by controlling the pH,[6] the method is uneconomical for the industrial preparation of L-ascorbic acid. The reason for this is that neither of the two required pentose sugars is readily accessible at the present time.

Another method that has been examined for synthesizing L-ascorbic acid involves the condensation of glyoxylic ester with L-threose in the presence of a base; instead of L-threose one can use its cyanohydrin which is prepared from L-xylose or L-lyxose.[7] As has been stated already, these two pentose sugars are inaccessible and so is L-threose, and hence this method of synthesizing L-ascorbic acid is likewise of no commercial value at present.

The most useful method at the present time is based upon the observation that an α-keto-β,γ-dihydroxy carboxylic acid (I) may be transformed into a compound having the ascorbic acid ring system (III) either directly by the agency of an acid or indirectly by first converting the acid into an ester (II) and then treating the latter with sodium methoxide.[8]

Researches on the large-scale preparation of L-ascorbic acid have, therefore, been directed mainly toward the development of methods for producing the requisite α-ketohydroxy acids, namely, 2-keto-L-gulonic and 2-keto-L-idonic acid. Although it is claimed that L-idonic acid may be oxidized to the corresponding 2-keto acid by a fermentation process[9] and that L-gulonic acid can be oxidized to a 2-keto acid by means of chromic acid,[10, 11]

[1] R. G. Ault, D. K. Baird, H. C. Carrington, W. N. Haworth, R. W. Herbert, E. L. Hirst, E. G. V. Percival, F. Smith, and M. Stacey, *J. Chem. Soc.* **1933**, 1419.
[2] T. Reichstein, A. Grüssner, and R. Oppenauer, *Helv. Chim. Acta* **16**, 1019 (1933).
[3] L. L. Salomon, J. J. Burns, and C. G. King, *J. Am. Chem. Soc.* **74**, 5161 (1952).
[4] J. K. Hamilton and F. Smith, *J. Am. Chem. Soc.* **74**, 5162 (1952).
[5] R. Weidenhagen, *Z. Wirtschaftgruppe Zuckerind.* **87**, 711 (1937).
[6] I. Stone, U. S. Pat. 2,206,374 (1940).
[7] B. Helferich and O. Peters, *Ber.* **70**, 465 (1937); German Pat. 637,448 (1936).
[8] T. Reichstein and A. Grüssner, *Helv. Chim. Acta* **17**, 311 (1934).
[9] U. S. Pats. 2,421,611 and 2,421,612 (1947).
[10] R. Pasternak and P. P. Regna, U. S. Pat. 2,153,311 (1939).
[11] R. Pasternak and P. P. Regna, U. S. Pat. 2,188,777 (1940).



these procedures are not so valuable from a commercial point of view as those utilizing L-sorbose. The latter, once a rare sugar, can be efficiently produced on a large scale by the bacterial oxidation of D-glucitol (sorbitol),[12, 13] which is readily obtainable by the catalytic hydrogenation of D-glucose.

Attention has therefore been focused on the oxidation of L-sorbose to 2-keto-L-gulonic acid. This transformation can be brought about directly with nitric acid[14-17] or with other oxidizing agents such as hypochlorite, peroxide, and permanganate at neutral pH[18]. Electrolytic oxidation of L-sorbose to 2-keto-L-gulonic acid has also been recommended.[19]

A better direct process seems to be that in which the L-sorbose is oxidized with air in the presence of a noble metal catalyst such as platinum at a neutral or slightly alkaline pH.[20, 21] The general field of catalytic oxidations of this type has not been investigated as much as it deserves for it not only holds out considerable promise in the case under discussion but it is worthy of wide application in organic chemistry.

The indirect transformation of L-sorbose into 2-keto-L-gulonic acid, by making use of a derivative in which only the CH_2OH group at C_1 is free to be oxidized, provides an excellent and probably the best approach to the manufacture of L-ascorbic acid. To achieve this, L-sorbose is converted into 2,3,4,6-diisopropylidene-L-sorbofuranose; analogous derivatives may be made with other carbonyl compounds such as cyclohexanone. Such

[12] P. A. Wells, J. J. Stubbs, L. B. Lockwood, and E. T. Roe, *Ind. Eng. Chem.* **29**, 1385 (1937).
[13] U. S. Pat. 2,121,533 (1938).
[14] W. N. Haworth, E. L. Hirst, J. K. N. Jones, and F. Smith, British Pat. 443,901 (1936).
[15] J. Overhoff and W. Huyser, U. S. Pat. 2,467,442 (1949).
[16] Dutch Pat. 59,301 (1947).
[17] Dutch Pat. 59,584 (1947).
[18] German Pat. 644,962 (1937).
[19] Danish Pat. 68,836 (1949).
[20] K. Heyns, *Ann.* **558**, 177 (1947).
[21] O. Dalmer and K. Heyns, German Pat. 692,897 (1940); U. S. Pat. 2,190,377 (1940)

derivatives are then oxidized with alkaline permanganate to give the dicarbonyl derivative of 2-keto-L-gulonic acid.[8, 22, 23] The free 2-keto acid may be generated by autohydrolysis or by means of dilute mineral acid, after which it may be converted into L-ascorbic acid by either of the two methods given above.

It has also been found possible to bring about the direct conversion of diisopropylidene 2-keto-L-gulonic acid[24-27] and its ester[28] into L-ascorbic acid. By choosing the right solvent, this ingenious device enables L-ascorbic acid to be crystallized directly from the reaction mixture.

Success is also said to attend the acid-catalyzed conversion of molten 2-keto-L-gulonic acid to L-ascorbic acid.[29]

IV. Biochemical Systems[1]

L. W. MAPSON

A. SYNTHESIS OF ASCORBIC ACID

Ascorbic acid is widely distributed throughout the animal and plant kingdoms. In animal tissue the highest concentrations are found in the suprarenal cortex, eye lens, and liver tissues; in plants it is most abundant in the most actively growing regions. Ascorbic acid occurs as such and in the oxidized form, dehydroascorbic acid. It has also been alleged to occur in a combined form as "ascorbigen," on the evidence that, when plant tissues are heated, more ascorbic acid may be extracted than if the tissue is extracted by acid in the cold.[1a, 2] This increase on heating has not been observed by all workers, however, and the phenomenon has been explained as being

[22] E. Boasson, S. Goldschmidt, and A. Middelbeek, Dutch Pat. 57,142 (1946).

[23] Swiss Pat. 174,080 (1935); U. S. Pat. 2,039,929 (1936); British Pat. 435,971 (1935).

[24] T. Reichstein, British Pat. 446,548 (1937).

[25] R. Pasternak and G. O. Cragwell, U. S. Pat. 2,185,383 (1940).

[26] V. M. Berezovskiĭ and L. J. Strel'chunas, *Zhur. Priklad. Khim.* **22**, 1113 (1949) [*C.A.* **45**, 5627 (1951)].

[27] H. W. Bassford, Jr., W. S. Harmon, and J. F. Mahoney, U. S. Pat. 2,462,251 (1949).

[28] A. H. Ruys and J. F. Lemmens, U. S. Pat. 2,491,933 (1950).

[29] Dutch Pat. 59,710 (1947).

[1] This section was prepared as part of the program of the Food Investigation Organisation of the Department of Scientific and Industrial Research, England.

[1a] B. Ahmad, *Nature* **136**, 797 (1935); E. W. McHenry and M. Graham, *ibid.* **135**, 871 (1935); E. J. Reedman and E. W. McHenry, *Biochem. J.* **32**, 85 (1938); H. Scarborough and C. P. Stewart, *ibid.* **31**, 1874 (1937); *Nature* **142**, 40 (1938).

[2] K. C. Saha, *J. Indian Chem. Soc.* **16**, 511 (1939); B. C. Guha and P. N. Sen-Gupta, *Nature* **141**, 974 (1938); J. C. Pal and B. C. Guha, *J. Indian Chem. Soc.* **16**, 481 (1939); P. N. Sen-Gupta and B. C. Guha, *ibid.* **16**, 496 (1939).

due to a more rapid inactivation of oxidase enzymes by heat than by the faulty techniques employed for extraction in the cold.[3-5] When proper precautions are observed to prevent oxidation of ascorbic acid, there is little evidence of any increase of ascorbic acid on heating; in fact the reverse is true. A bound form of ascorbic acid was alleged to have been isolated from cabbage and cauliflower,[6] but later workers have not confirmed this.[7]

1. Site of Formation

It is not known whether ascorbic acid is synthesized in any one tissue or is capable of being synthesized by all tissues in either the plant or animal. In animal tissues it has been suggested that ascorbic acid is synthesized in both intestinal[7] and liver tissue,[8] although the evidence so far available favors the liver as the site of formation. The recent alternative suggestion that the suprarenal gland may be the chief organ in the animal synthesizing ascorbic acid is based on the experimental observation that the ascorbic acid excreted by rats after adrenalectomy falls to very low values.[9] In intact animals the greatest excretion of the vitamin occurred just prior to sexual maturity, suggesting that the activity of endocrine glands other than the adrenal may also influence the synthesis. Histochemical studies of the animal cell have shown that ascorbic acid appears in highest concentration in the Golgi apparatus and mitochondria,[10] and in scorbutic animals these constituents of the cell show changes which are rectified on the addition of ascorbic acid.[11] There is no evidence to suggest, however, that ascorbic acid is synthesized in these structures.

All plant tissues contain ascorbic acid except woody tissues; seeds are also devoid of it, but the vitamin is formed in the early stages of germination.[12] In the later development of the plant, ascorbic acid is formed continually in the green organs,[13, 14] and numerous attempts have been made to identify the chloroplasts as the sites of ascorbic acid synthesis. The reduction of $AgNO_3$ is said to occur only in the chloroplasts,[15] and this action

[3] K. Wacholder and A. O. Kreut, *Hoppe-Seyler's Z. physiol. Chem.* **264**, 254 (1940).
[4] A. Fujita and T. Ebihara, *Biochem. J.*, **301**, 229 (1939).
[5] L. J. Harris and M. Olliver, *Biochem. J.* **36**, 155 (1942).
[6] B. Ghosh and B. C. Guha, *J. Indian Chem. Soc.* **16**, 505 (1939).
[7] F. Widenbauer and K. Koschorrech, *Biochem. Z.* **291**, 209 (1937).
[8] M. N. Rudra, *Nature* **144**, 868 (1939).
[9] M. E. Dumm and E. P. Ralli, *Endocrinology* **45**, 188 (1949).
[10] G. Bourne, *Australian J. Exptl. Biol. Med. Sci.* **13**, 239 (1935).
[11] A. Miwa, *Oriental J. Diseases Infants* **26**, 3 (1939).
[12] L. J. Harris and S. N. Ray, *Biochem. J.* **27**, 580 (1933).
[13] A. Giroud, R. Ratsimamanga, and C. P. Leblond, *Compt. rend. soc. biol.* **117**, 612 (1934).
[14] L. Randoin, A. Giroud, and C. P. Leblond, *Bull. soc. chim. biol.* **17**, 1649 (1935).
[15] A. Giroud, C. P. Leblond, and R. Ratsimamanga, *Compt. rend. soc. biol.* **117**, 614 (1934).

has been attributed to ascorbic acid. Improved tests have shown, however, that the reduction of the silver reagent may occur in the colorless stroma of the plastid, but it is extremely doubtful if the reaction can be taken as being specific for the presence of ascorbic acid. As an alternative explanation of the reaction it has been claimed that the reduction of $AgNO_3$ by green tissue is in effect a photolysis of $AgNO_3$ in which chlorophyll acts as an optical sensitizer, irrespective of the presence of ascorbic acid.[16]

It also seems clear that chlorophyll is not necessary for the synthesis of ascorbic acid, and any apparent relationship between the formation of ascorbic acid and of chlorophyll is probably the result of conditions which favor the development of both substances. Ascorbic acid may accumulate in plant tissue independently of the formation of chlorophyll; e.g., the corolla lacks chlorophyll but contains more ascorbic acid than the calyx.[17] The best evidence, however, that ascorbic acid may be synthesized in the absence of chlorophyll is the fact that seeds germinated in complete darkness produce ascorbic acid but not chlorophyll.

2. Formation from Sugars

Interpretation of studies on the synthesis of ascorbic acid is complicated by the fact that an increase in the concentration of the vitamin in the cell may, as far as our present knowledge indicates, arise either (a) by an increase in the rate at which it is synthesized or (b) by a decrease either in the rate at which it is oxidized to dehydroascorbic acid or in the rate at which the latter is irreversibly converted into 2,3-diketogulonic acid. It is usually extremely difficult to decide between the two possibilities. In other words, the presence of ascorbic acid in higher concentration in some tissues than in others may be related to conditions favoring stability rather than to those favoring synthesis; in fact its presence in a cell does not necessarily mean that it was synthesized there at all.

The mechanism of the synthesis of ascorbic acid, whether in plants or in animals, is not understood. Studies on this subject have been concerned with supplying possible precursors to the plant or animal and to ascertain the effect of these substances on the synthesis. Since seeds are devoid of ascorbic acid, but produce it during germination, this phase of plant life has been examined by many workers as offering the best material for study. Seedlings grown in either water or inorganic mineral cultures form ascorbic acid in conditions in which photosynthesis cannot occur. Under such conditions the synthesis must take place at the expense of a carbon source derived from the reserves of the seed. Excised pea embryos grown on sterile synthetic media form ascorbic acid if supplied with certain hexose sugars. Of these, fructose, mannose, glucose, and galactose have been found to be

[16] R. J. Goutheret, Thèse Sciences, Paris (1935).
[17] M. E. Reid, *Am. J. Bot.* **24**, 445 (1937).

good precursors. Substances such as methyl glyoxal, pyruvate, glycerophosphate, glutamate, and pentose sugars were ineffective. Glycerol and disaccharides, e.g., sucrose, were also found to act as precursors.[18]

Further studies with seedlings have indicated a close correlation between the synthesis of hexose sugars during germination and that of ascorbic acid, a correlation which holds when the seedlings are grown under conditions in which the amount of ascorbic acid synthesized is altered by altering the nutrient salts supplied.[19, 20] Furthermore, it has been shown that the effect of different nutrient salts is due to their effect on the pH of the cell sap. Salts which decrease the pH decrease and salts which increase it increase the synthesis of ascorbic acid. Similarly a shift of the pH of the cell sap to more alkaline values increases, and a shift to more acid values decreases the efficiency of the conversion of hexose sugars to ascorbic acid.

The formation of ascorbic acid from hexose sugars has also been reported to occur in animal tissues. Dextrose has been found to increase the ascorbic acid content of slices of intestinal tissue, but not of liver, spleen, stomach, or brain.[21] In these experiments, however, the vitamin was determined by iodine titration, an unspecific method. The synthesis has also been observed in spleen, liver, and cardiac muscle tissue *in vitro* from mannose. The mechanism of the transformation is claimed to be enzymatic in nature, the enzyme being a dehydrogenase.[22] So far these results have not been confirmed by other workers.

Evidence along somewhat different lines has been produced by a study of the increased excretion of ascorbic acid by the rat after administration of certain drugs, notably the terpene-like cyclic ketones.[23, 24] These drugs appear to stimulate the synthesis of ascorbic acid and are themselves excreted in combination with glycuronic acid. Ascorbic acid appears as such in the urine but not in combination with any of the drugs. It is of interest that there appears to be a correlation between the formation of glycuronic acid needed for detoxication and the formation of ascorbic acid, and we may speculate whether in the formation of glycuronic acid metabolic intermediates are formed which also lead to an increased synthesis of the vitamin. The increased synthesis of ascorbic acid appears to occur in the liver and kidney, for liver and kidney slices from chloretone-treated animals continue to synthesize ascorbic acid in greater quantity than the corres-

[18] S. N. Ray, *Biochem. J.* **28**, 996 (1934).
[19] L. W. Mapson and E. M. Cruickshank, *Biochem. J.* **41**, 197 (1947).
[20] L. W. Mapson, E. M. Cruickshank, and Y.-T. Chen, *Biochem. J.* **45**, 2, 171 (1949).
[21] F. Widenbauer and K. Koschorrech, *Biochem. Z.* **291**, 209 (1937).
[22] B. C. Guha and A. R. Ghosh, *Nature* **134**, 739 (1934); **135**, 871 (1935).
[23] H. E. Longenecker, R. R. Musulin, R. H. Tully, and C. G. King, *J. Biol. Chem.* **129**, 445 (1939).
[24] J. Mosonyi, *Hoppe-Seyler's Z. physiol. Chem.* **230**, 240 (1934).

ponding tissues from control animals. The synthesis was greatest when the tissues were supplied with a mixture of pyruvate, DL-glyceraldehyde, and hexose diphosphate.[25] Mannose, glucose, and 2-ketogulonic acid were ineffective.

The close link in animal and plant tissue between hexose sugars and ascorbic acid has stimulated speculation as to the method by which the transformation of one to the other is effected. A direct conversion of D-glucose to L-ascorbic acid without breaking the carbon chain of the sugar involves the inversion of the configuration of the groups in carbon atom 5. A simple scheme based on known chemical and biochemical reactions can be suggested, viz., D-glucose → sorbitol → L-sorbose → 2-ketogulonic acid → L-ascorbic acid.

L-Sorbose has been reported by one worker as a precursor of ascorbic acid,[26] but this has not been substantiated by others.[27]

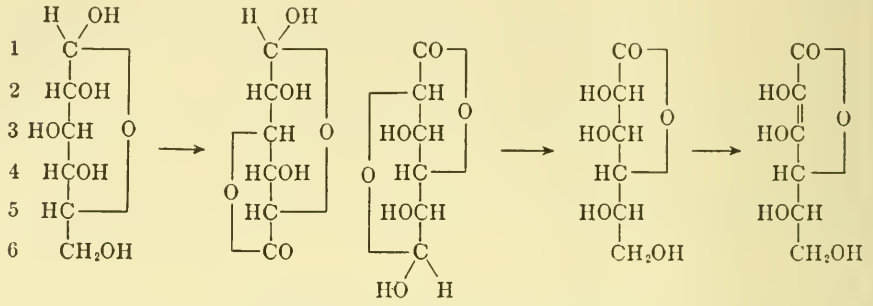
Neither sorbitol nor 2-keto-L-gulonic acid when supplied to seedlings increased the amount of ascorbic acid synthesized.[28] The formation of ascorbic acid from 2-keto-L-gulonic acid has been reported to occur in *Aspergillus niger*,[28] and methyl-2-keto-L-gulonate has been reported as having antiscorbutic acitivity,[29] though the free acid is inactive.

The most direct evidence bearing on the synthesis of ascorbic acid from glucose has been obtained by means of radioactive tracer technique. When D-glucose, labeled uniformly in all positions, is fed to chloretone-treated rats, it is followed by the excretion of ascorbic acid labeled uniformly in all positions.[30] The suggestion has been made that the carbon chain of D-glucose is not broken before being converted into ascorbic acid, or that, if so, the fragments are recombined without any major differential dilution effect.

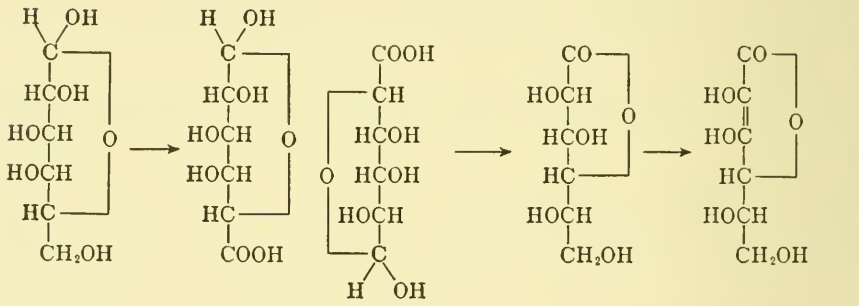
Two papers have recently appeared which throw considerable light on the mechanism of synthesis. Using the radioactive tracer technique, Horowitz *et al.*[31] have shown that feeding of D-glucose labeled with C^{14} at carbon atom 1 produces L-ascorbic acid labeled only at position 6. Even more illuminating is the work of Isherwood *et al.*,[32a] who have shown that in both plants and animals D-glucurono-γ-lactone and L-gulono-γ-lactone on the one hand and D-galacturonic acid methyl ester and L-galactono-γ-lactone on the

[25] C. V. Smythe and C. G. King, *J. Biol. Chem.* **142**, 529 (1942).
[26] G. V. Sztaneczy, *Biochem. Z.* **295**, 369 (1938).
[27] Y.-T. Chen, Ph.D. Thesis, Cambridge (1950).
[28] A. Galli, *Ber. schweiz. bot. Ges.* **56**, 113 (1946).
[29] T. Reichstein and V. Demole, *Festschr. Emil Barell*, Basel (1936); G. Lorenzini and A. Corbellini, *Arch. inst. biochem. Ital.* **10**, 131 (1938).
[30] S. S. Jaekel, E. H. Mosbach, J. J. Burns, and C. G. King, *J. Biol. Chem.* **186**, 569 (1950).
[31] H. H. Horowitz, A. P. Doerschuk, and C. G. King, *J. Biol. Chem.* **199**, 193 (1952).
[32a] F. A. Isherwood, Y.-T. Chen, and L. W. Mapson, *Nature* **171**, 348 (1953).

other produce L-ascorbic acid when fed to cress seedlings or injected into rats. The evidence from this work indicates that L-ascorbic acid may be formed by a sequence of reactions as follows.



D-Glucose — Alternative forms of writing D-glucurono-γ-lactone to show inversion of configuration — L-Gulono-γ-lactone — L-Ascorbic acid



D-Galactose — Alternative forms of writing D-galacturonic acid to show inversion of configuration — L-Galactono-γ-lactone — L-Ascorbic acid

A fundamental feature of both reaction chains is the change from the D to the L series with inversion of the whole molecule at the second step; which of these reaction sequences is the more important in the synthesis of L-ascorbic acid in the plant or animal is not at present known.

Confirmation that the synthesis of ascorbic acid *in vivo* does in fact proceed along these or very similar pathways has been forthcoming by recent observations[32b] that both D-galacturonic acid methyl ester and L-galactono-γ-lactone are converted *in vitro* to L-ascorbic acid by extracts from plant tissues. The oxidation of L-galactono-γ-lactone to L-ascorbic acid was found to be catalyzed by the cytoplasmic particles (mitochondria) of the cell prepared from germinating pea seeds. The observations also showed

[32b] L. W. Mapson, F. A. Isherwood, and Y.-T. Chen, *Biochem. J.* (1953, in press).

that the cytochrome system was involved, for the reaction was inhibited by cyanide, by azide, and by carbon monoxide in the dark, the effect of the latter being reversed in light. Oxygen appeared to be necessary as the terminal hydrogen acceptor. There was no evidence to suggest the necessity for the participation of compounds containing high-energy phosphate bonds in the formation of the vitamin from L-galactono-γ-lactone. Under the same conditions, however, neither L-gulono-γ-lactone nor D-glucurono-γ-lactone was converted to L-ascorbic acid. The reason for this remains obscure, since L-gulono-γ-lactone is converted to L-ascorbic acid by intact pea seedlings, although the formation of L-ascorbic acid in this case is much less (five to six times) than with L-galactono-γ-lactone.

3. Dependence of Biosynthesis on External Factors

a. Salts

Many workers have studied the effect on ascorbic acid in plants of supplying different fertilizers. The evidence obtained from these studies has on the whole been conflicting and difficult to interpret. Plants receiving a full nutrient supply have been reported as containing more ascorbic acid than plants on a lower nutrient plane.[33] Ascorbic acid in potatoes was found to be increased by mixtures of salts rich in nitrogen or phosphorus but to be decreased by mixtures containing potassium or calcium. Salts such as KCl, $Ca_3(PO_4)_2$, and $Ca(NO_3)_2$ were found to promote the formation of ascorbic acid in pea seedlings when a properly balanced mixture was supplied.[34]

The effect of nitrogenous fertilizers on the synthesis of ascorbic acid has been extensively studied, but the results obtained have again been contradictory. In some cases the fertilizers lowered,[35] and in others they increased, the formation of ascorbic acid in plant tissues.[36] The ascorbic acid content of plants fertilized with ammonium nitrate varied with the amount of the fertilizer supplied. With suboptimum supplies, the ascorbic acid content of adult leaves decreased, but with supraoptimum amounts the reverse occurred. The decrease in ascorbic acid was believed to be connected with osmotic changes.[37] Several workers have found that the supply of rich

[33] A. Giroud, R. Ratsimamanga, C. P. Leblond, H. Chalopin, and M. Rabinowicz, *Bull. soc. chim. biol.* **18**, 573 (1936).

[34] S. Von Hausen, *Ann. Acad. Sci. Fennicae, Ser.* **A46**, 134 (1935).

[35] K. Wacholder and K. Nehring, *Bodenkunde u. Pflanzenernähr.* **16**, 245 (1940); T. Mott, *Angew. Chem.* **50**, 75, 1937; C. R. Fellers, R. E. Young, P. Isham, and J. Claque, *Proc. Am. Soc. Hort. Sci.* **31**, 145 (1934).

[36] I. Hoffman, W. Krauss, and R. Washburn, *Ohio Agr. Expt. Sta. Bull.* **592**, 66 (1938); R. Balks and E. Pommer, *Bodenkunde u. Pflanzenernähr.* **9**, 724 (1938); C. Pfaff and G. Pfutzer, *Angew. Chem.* **50**, 179 (1937).

[37] B. Åberg and I. Ekdahl, *Physiol. Plantarum* **1**, 290 (1948).

nitrogenous fertilizers to fruit trees lowered the ascorbic acid content of the fruit.[38] Observations on grapefruit indicated that there was an inverse relationship between the nitrogen and ascorbic acid content.[39] The effect of nitrogenous fertilizers may, however, be overshadowed by the effect of other environmental factors such as soil or light intensity.[40, 41] Moreover, the effect of nitrogen per se is difficult to assess, since fertilizers rich in this element inevitably affect the size of crop, the amount of foliage, etc., all of which have important indirect effects on the synthesis of the vitamin. It has also been suggested that the higher rate of respiration usually induced by higher nitrogen supply may also influence the concentration of ascorbic acid by increasing its rate of consumption,[42] or by depressing the photosynthetic products available for synthesis by stimulating other competitive synthetic processes.[43]

The form in which nitrogen is supplied to the plant is equally important, although contrary effects have been reported. Some reports indicate that plants supplied with nitrate contain less ascorbic acid than those grown on ammonium salts.[44] In other studies the reverse has been reported. With cress seedlings the effect of ammonium nitrogen depends on the type of ammonium salt fed. Ammonium sulfate and chloride depress the synthesis of ascorbic acid, but ammonium bicarbonate, acetate, or succinate have relatively little effect. Of a large number of salts tested, it has been shown that they influence the synthesis of ascorbic acid by virtue of their effect in altering the pH of the cell sap. When the cation of the salt is NH_4 and combined with a non-utilizable anion, there is a marked depressant effect, which is reduced when the cation is combined with a utilizable anion. Conversely, calcium or potassium salts, when combined with a utilizable anion, e.g., nitrate, succinate, bicarbonate, or acetate, increase the synthesis to a greater extent than when they are supplied in combination with non-utilizable anions, e.g., sulfate or chloride.[19]

[38] W. W. Jones and E. R. Parker, *Proc. Am. Soc. Hort. Sci.* **50**, 195 (1947); S. H. Wittwer and A. D. Hibbard, *Proc. Am. Soc. Hort. Sci.* **49**, 116 (1947). T. Wallace and S. S. Zilva, *Biochem. J.* **27**, 693 (1933); W. Kessler, *Gartenbauwiss.* **13**, 619 (1939); A. H. Finch, W. W. Jones, and C. W. Van Horn, *Proc. Am. Soc. Hort. Sci.* **46**, 314 (1945).

[39] W. W. Jones, C. W. Van Horn, A. H. Finch, M. C. Smith, and E. Caldwell, *Science* **99**, 103 (1944).

[40] K. J. Kasihka, L. T. Dudgeon, and H. M. Hauch, *J. Agr. Research* **68**, 49 (1944).

[41] K. C. Hamner, L. Bernstein, and L. A. Maynard, *J. Nutrition* **29**, 85 (1945).

[42] H. Burstom, *Arkiv. Botan.* **32A**, 7:1 (1945); G. T. Nightingale, *Botan. Rev.* **3**, 85 (1937); **14**, 185 (1948).

[43] B. Åberg and I. Ekdahl, *Physiol. Plantarum* **1**, 290 (1948).

[44] W. W. Jones, W. P. Bitters, and A. H. Finch, *Proc. Am. Soc. Hort. Sci.* **45**, 1 (1944); A. H. Finch, W. W. Jones, and S. W. Van Horn, *ibid.* **46**, 314 (1947); S. H. Wittwer, R. A. Schroeder, and W. A. Albrecht, *Soil Sci.* **59**, 329 (1945).

b. Manganese

Manganese has been reported as being a necessary cofactor in the synthesis of ascorbic acid. Seedlings of *Avena*, *Triticum*, *Hordeum*, *Phascolus*, *Mungo*, and *Cicer arietinum* were found to contain more ascorbic acid when grown in the presence of manganese salts than when grown in their absence.[45] The ascorbic acid content of tomatoes grown in soils low in manganese was found to be lower than that of similar plants grown in soils with higher manganese contents.[46] This work was extended to animal tissues, and it was reported that with both rat and guinea pig liver tissues the formation of ascorbic acid was increased *in vitro* if the tissue was supplied with mannose, glucose or galactose, and manganese salts; the increase did not occur if sugar alone was supplied.[47] With guinea pig liver a higher concentration of manganese was found to be essential. Similar findings were reported from experiments when rats or guinea pigs were injected with the sugar and manganese salt.

These results with animal tissue have not, however, been confirmed by later workers.[48] When scorbutic guinea pigs were injected with both mannose and manganese salts there was no evidence, either by the phosphatase test or by an analysis of tooth structure, of any protection against scurvy, which developed as quickly in injected animals as in control animals. The hypothesis that manganese is an essential cofactor in the synthesis of ascorbic acid needs further investigation.

c. Molybdenum

The possible role of molybdenum has recently been emphasized.[49] It has been shown that the tissues of plants deficient in this element are low in ascorbic acid. Neither disparity in growth nor lack of chlorophyll could account for this. It is too early to decide whether molybdenum affects the synthesis of ascorbic acid directly or whether the lack of it leads to a disturbance in the normal ascorbic acid-dehydroascorbic acid equilibrium in the cell. The role of molybdenum in higher plants is thought to be closely connected with the assimilation of nitrate nitrogen;[50] molybdenum requirements appear to be much reduced when nitrogen is supplied as ammonium salts.[51] It may therefore function in the processes concerned with the reduction of nitrate. The suggestion that ascorbic acid may also be associated

[45] M. N. Rudra, *Nature* **141**, 203 (1938); *Biochem. Z.*, **301**, 238 (1939).
[46] J. B. Hester, *Science* **93**, 401 (1941).
[47] M. N. Rudra, *Nature* **144**, 868 (1939).
[48] J. T. Skinner and J. S. McHargue, *Am. J. Physiol.* **145**, 566 (1946).
[49] E. J. Hewitt, S. C. Agarwala, and E. W. Jons, *Nature* **166**, 1119 (1950).
[50] E. J. Hewitt and E. W. Jons, *J. Pomol. Hort. Sci.* **23**, 254 (1947).
[51] E. G. Mulder, *Plant and Soil* **1**, 94 (1948).

in the cell with the reduction of nitrate is not new. It has recently been shown that seedlings given nitrate may be unable to grow after removal of cotyledons or endosperm unless ascorbic acid, glutathione, or other reducing substances are added, whereas excised seedlings given nitrogen in the form of ammonium salts continue normal development in the absence of any supplements.[52]

d. Light

Light has a beneficial effect upon the production of ascorbic acid in plants.[53] The concentration of ascorbic acid in plant leaves fluctuates considerably during the day, the maximum being reached in the forenoon.[54] A fall in the ascorbic acid content of plants has been observed when they were transferred from light to dark, and also when parts of the plant were shaded.[55] With fruits the concentration of ascorbic acid varies with the degree to which the fruit is exposed to sunlight.[56] A direct relationship between the ascorbic acid formed and the light intensity has also been observed with turnip leaves.[57] The effect of light varies with the wavelength; red light is the most effective both for the production of ascorbic acid and for photosynthesis,[58] whereas the rays of the blue-violet end of the spectrum have no influence.[59] Certain experimental results support the view that the synthesis of ascorbic acid is linked with photosynthesis. Seedlings deprived of their reserves and grown in light without carbon dioxide (no photosynthesis) show a fall in the concentration of ascorbic acid in their tissues.[60] Rhizomes of Stachys which had developed chlorophyll under the influence of light contained more ascorbic acid than controls kept in the dark.[61] With cut discs of turnip leaves the ascorbic acid increased in the

[52] A. I. Vitanem and S. S. Von Hausen, *Nature* **163**, 482 (1949).

[53] V. G. Heller, *J. Biol. Chem.* **76**, 499 (1928); T. Matsuoka, *Mem. Coll. Agr. Kyoto Univ.* **9**, 1 (1930); **24**, 37 (1932); **35**, 1 (1935); W. Y. Lee and B. E. Read, *J. Chinese Chem. Soc.* **4**, 208 (1936); M. E. Reid, *Am. J. Bot.* **24**, 445 (1937).

[54] H. G. Moldtman, *Planta* **30**, 297 (1939); A. M. Smith and J. Gillies, *Biochem. J.* **34**, 1312 (1940).

[55] P. L. Harding and E. E. Thomas, *J. Agr. Research* **64**, 57 (1942); P. L. Harding, J. R. Winston, and D. F. Fisher, *Proc. Am. Soc. Hort. Sci.* **36**, 358 (1938); W. Kessler, *Gartenbauwiss.* **13**, 619 (1939); J. P. McCollum, *Proc. Soc. Hort. Sci.* **45**, 382 (1944).

[56] N. V. Veselkine, V. N. Lubimenko, Z. P. Boulgakova, V. V. Tikalsskaia, and P. S. Engel, *Bul. Inst. Sci. Lesshaft.* **17**, 389 (1934); F. Wokes and J. G. Organ, *Biochem. J.* **37**, 259 (1943); K. C. Hamner, L. Bernstein, and L. Maynard, *J. Nutrition* **29**, 85 (1945)

[57] G. F. Somers, W. C. Kelly, and K. C. Hamner, *Arch. Biochem.* **18**, 59 (1948).

[58] T. Sugawara, *Japan. J. Bot.* **10**, 325 (1939).

[59] B. Åberg, *Ann. Roy. Agr. Coll. Sweden* **15**, 239 (1945).

[60] K. Weissenböck and M. Weissenböck, *Protoplasma* **34**, 585 (1940).

[61] F. Weber, *Protoplasma* **34**, 135 (1940).

light, and moreover carbon dioxide was necessary for its accumulation.[57] In general, factors which favor photosynthesis also favor biosynthesis of ascorbic acid, although the connection between these metabolic activities may be indirect. If sugar serves as the precursor of ascorbic acid it is only natural that its formation should be accelerated under conditions in which sugar is being formed. This does not exclude the possibility that other, more active intermediates may be formed during photosynthesis. Åberg[59] has suggested that there are two different routes for ascorbic acid synthesis in the plant: (a) a synthesis independent of light, which occurs mainly in germinating seeds and (b) a light-conditioned synthesis which is connected with the assimilation of carbon dioxide.

e. Relation between Synthesis of Carotenoids and Ascorbic Acid

Fruits richest in carotenoids are also usually rich in ascorbic acid.[62] Seedlings grown at low temperature were found to form much ascorbic acid and also carotenoids, but little chlorophyll.[61] With cress seedlings the effect of different nutrients salts had a similar effect on the synthesis of both ascorbic acid and carotene.[19] It is difficult, however, to establish a direct chemical relationship on the one hand, or on the other to decide whether this parallelism is pure chance. The suggestion[62] has been made that carotene may protect ascorbic acid from oxidation, thus causing an accumulation of the vitamin in the cell.

f. Relation to Sucrose

In detached leaves kept in the dark the ascorbic acid content falls; this fall may be arrested and an increase of ascorbic may in some cases actually occur by floating the leaves in a 10% sucrose solution.[63] With submerged leaves in water the fall of ascorbic acid is not arrested but even accentuated, but there is no indication as to whether this treatment increases the sucrose content inside the cell. Other evidence, however, points to some relation between sucrose and ascorbic acid. With detached leaves placed in the dark and supplied with weak solutions of iodoacetate, fluoride, or arsenite, a marked fall in the ascorbic acid of the leaf occurred, and the fall was accompanied or even slightly preceded by a parallel fall in sucrose.[64] The same process appears to go on in leaves left in the dark and supplied with water alone, although the rate of change is much slower. A correlation between ascorbic acid and sucrose content has also been observed in potato tubers subjected to temperatures of $-0.8°$ to $+1°$ to induce sweetening.[65] Under

[62] A. Giroud, *Protoplasma* (*Monogr.*) **16**, 1 (1938); A. Giroud, R. Ratismamanga, R. Leblond, H. Chalopin, and M. Rabinowicz, *Bul. soc. chim. biol.* **18**, 573 (1936).
[63] B. Åberg, *Physiol. Plantarum* **2**, 164 (1949).
[64] L. W. Mapson and J. Barker, Unpublished data.
[65] J. Barker and L. W. Mapson, *New Phytologist* **49**, 283 (1950).

these conditions there is an actual increase in both the concentration of sucrose and ascorbic acid. Conditions which promote the synthesis of sucrose therefore appear also to favor an increase in the concentration of ascorbic acid. It is still not clear, however, whether the action is one of increased synthesis or one in which the rate of loss of ascorbic acid is reduced. A direct chemical relationship between sucrose and ascorbic acid is difficult to visualize; it is conceivable that some essential component in the synthesis of sucrose may be concerned either directly in the synthesis of ascorbic acid, or in a sequence of reactions which stabilize the vitamin in the cell.

4. Synthesis of Ascorbic Acid in Vitamin-Deficient Animals

Work over the past ten years has suggested the possibility that vitamin A is necessary for the synthesis of ascorbic acid in animals. Single or repeated depletion of vitamin A caused a fall in the ascorbic acid content of the tissues of rats,[66] and with rats fed with graded doses of vitamin A it was found that the ascorbic acid concentration in the plasma ran parallel with the vitamin A intake.[67] The observation that animals deficient in vitamin A excrete less ascorbic acid than the non-deficient animals[68] was in line with the above results and suggested that the absence of vitamin A decreases the concentration of ascorbic acid in the tissues by interfering with its synthesis and not by causing an excessive loss from the kidney. Observations with calves and horses suffering from vitamin A deficiency showed that the lowered level of ascorbic acid in plasma and cerebrospinal fluid could be raised by the administration of vitamin A.[69] These observations on the rat were generally confirmed, but the interpretation that this represented a decreased synthesis of ascorbic acid due directly to a lack of vitamin A per se was negatived.[70] It was shown that the lowered concentration of ascorbic acid in the tissues of animals deprived of vitamin A could be accounted for solely by their lowered food intake. Moreover the rise in the urinary excretion of ascorbic acid following the administration of chloretone, although less in vitamin A-deficient rats than in non-deprived rats, was eliminated if the food intake of the latter was restricted to that of the deficient animal. No specific relationship was found between the rat's resources of vitamin A and its capacity to synthesize ascorbic acid.

Observations that a deficiency of vitamin produces scurvy-like alterations

[66] B. Sure, R. M. Theis, and R. T. Harrelson, *J. Biol. Chem.* **129**, 245 (1939).
[67] T. S. Sutton, H. Kaeser, and S. Hansard, *J. Biol. Chem.* **144**, 183 (1942).
[68] P. D. Boyer, P. H. Phillips, W. D. Pounden, C. Jenson, I. W. Rupel, and M. E. Nesbit, *J. Nutrition* **23**, 525 (1942).
[69] P. H. Phillips, N. S. Lundquist, and P. D. Boyer, *J. Dairy Sci.* **24**, 977 (1941).
[70] L. W. Mapson, and S. E. Walker, *Brit. J. Nutrition* **2**, 1 (1948).

in teeth[71] are also suspect, since it is well established that a deficiency of vitamin A leads to marked degenerative changes in the dentine and enamel of teeth;[72] furthermore the histological changes observed in vitamin A deficiency could not be cured by ascorbic acid, but only by vitamin A.

The evidence would therefore suggest that the lowered ascorbic acid content of tissues of vitamin A-deficient animals is due to inanition associated with the deficiency rather than to any specific effect of vitamin A on the synthesis of ascorbic acid.

The synthesis of ascorbic acid in animals has also been linked with thiamine and riboflavin[73, 74] With both these deficiencies the ascorbic acid content of the tissue of rats and mice has been shown to be low. The stimulation of synthesis shown by a normal animal after chloretone treatment was not observed in animals deficient in either thiamine or riboflavin.[74] The claim was made that these observations were directly due to a lack of these essential nutrients and not due to inanition. The suggestion that ascorbic acid may be synthesized in the narcotized animal from pyruvate and that the process requires the participation of both thiamine and riboflavin is of interest.

5. Glucoascorbic Acid and Synthesis of Ascorbic Acid

The report that a scorbutic-like condition in the rat, an animal which normally synthesizes its own ascorbic acid, could be induced by the administration of glucoascorbic acid,[75] an analog of ascorbic acid, did much to enliven interest in this substance as a means of throwing more light on the mechanism of the synthesis. Subsequent studies, however, have shown that the symptoms produced by glucoascorbic acid are not identical with those of scurvy;[76] the condition cannot be cured by feeding ascorbic acid, and there is no antagonism between these two substances when judged either by histological examination of teeth in guinea pigs or by the changes in serum phosphatase.[77] Glucoascorbic acid appears to produce toxic symptoms which are not due to any interference in the synthesis or functioning of L-ascorbic acid but are due to the toxic action of the substance itself.[78]

[71] G. Jonnson, A. L. Obel, and K. Sjöberg, *Z. Vitaminforsch.* **12**, 300 (1942); **15**, 115 (1945).

[72] S. B. Wolbach and P. Howe, *Am. J. Pathol.* **9**, 275 (1933); P. Boyle, *J. Dental Research* **13**, 39 (1933); H. Mellanby, *Brit. Dental J.* **67**, 187 (1939).

[73] E. L. Kennaway and M. E. Daff, *Brit. J. Exptl. Pathol.* **27**, 63 (1946).

[74] S. C. Roy, S. K. Roy, and B. C. Guha, *Nature* **158**, 238 (1946).

[75] D. W. Wolley and L. O. Krampitz, *J. Exptl. Med.* **78**, 353 (1943).

[76] R. J. Gorlin, *J. Dental Research* **29**, 208 (1950).

[77] B. S. Gould, *Arch. Biochem.* **19**, 1 (1948).

[78] W. G. Shafer, *J. Dental Research* **29**, 831 (1950).

B. FUNCTION OF ASCORBIC ACID IN BIOCHEMICAL SYSTEMS

The most obvious property of ascorbic acid is the ease with which it may be oxidized and reversibly reduced, and the possibility has led many investigators to attempt to show that this is its action *in vivo*. From the fact that both the reduced and the oxidized forms are usually found in animal and plant tissues and that its occurrence coincides quite generally with tissues possessing high metabolic activity, it seems a priori probable that it must possess properties which permit it to play an important role in respiration. The demonstration by Szent-Györgyi (1931),[79] and since then by many other workers, that accompanying ascorbic acid in plant tissues there is an enzyme, ascorbic oxidase, capable of catalyzing a direct reaction between ascorbic acid and molecular oxygen gave further impetus to the idea that ascorbic acid may act as a respiratory catalyst, acting in a similar capacity to that of cytochrome. In animal tissues it is true that no specific oxidase catalyzing the direct oxidation of ascorbic acid has as yet been isolated. Nevertheless, as will be shown later, other enzyme systems are present in animal tissues which are capable indirectly of catalyzing the oxidation of ascorbic acid. Neither do all plant tissues contain ascorbic oxidase, yet all plants contain oxidase enzymes which can indirectly catalyze the oxidation of ascorbic acid.

1. Systems Catalyzing the Oxidation of Ascorbic Acid

Ascorbic acid can be reversibly oxidized with extreme ease by a number of reagents. Both copper and iron salts are known to catalyze its oxidation,[80] and the oxidative activity of many tissues may, in part, be attributed to this cause, despite the fact that the activity of these metals is reduced by other constituents such as glutathione, amino acids, or proteins.[81] Other substances, the hemochromagens,[82] have also been shown to be good catalysts. Similarly, many quinones are capable of oxidizing it, and these substances may be formed from a variety of compounds present in both animal and plant tissues.

In addition to these non-enzymic catalysts, others of an enzymic nature are known to exist in both plant and animal tissues. There are at least five oxidases present in the tissues of higher plants that could be responsible for the entry of oxygen into the respiratory system. These are ascorbic

[79] A. Szent-Györgyi, *J. Biol. Chem.* **90**, 385 (1931).

[80] H. von Euler, K. Myrbäck, and H. Larson, *Hoppe-Seyler's Z. physiol. Chem.* **245**, 217 (1933); C. M. Lyman, M. O. Schultze, and C. G. King, *J. Biol. Chem.* **118**, 757 (1937); L. W. Mapson, *Biochem. J.* **39**, 228 (1945).

[81] E. S. G. Barron, A. G. Barron, and F. Klemperer, *J. Biol. Chem.* **116**, 563 (1936).

[82] E. S. G. Barron, R. H. De Meio, and F. Klemperer, *J. Biol. Chem.* **112**, 625 (1936); R. Lemberg, B. Cortis-Jones, and M. Norrie, *Biochem. J.* **32**, 149 (1938).

oxidase, polyphenoloxidase, cytochrome oxidase, laccase, and peroxidase. All these enzymes are capable of catalyzing the direct or indirect oxidation of ascorbic acid to dehydroascorbic acid. Thus, ascorbic acid is oxidized by the catechol-catechol oxidase system, by the cytochrome c-cytochrome oxidase system, by the flavone-peroxidase system, and by laccase and ascorbic oxidase. All these enzymes are proteins containing copper or iron as an integral part of their active prosthetic groups.

The mode and pathway of the oxidation of ascorbic acid occurs in two stages. The primary reversible oxidation product is dehydroascorbic acid, which is the lactone of 2,3-diketogulonic acid: the second stage of oxidation involves a cleavage of diketogulonic acid with the production of oxalic and L-threonic acids.[83] The conversion of dehydroascorbic acid into 2,3-diketogulonic acid is dependent on pH;[84] in neutral or alkaline solution this reaction is very rapid. The enzymic systems present in plant or animal tissues carry the oxidation only as far as dehydroascorbic acid. Little is known of any enzyme system affecting conversion of dehydroascorbic acid to 2,3-diketogulonic acid, although indication of the presence in wheat tissue of a thermolabile inhibitor of this reaction has been reported.[85] Thermostable substances such as borate[86] or cyanide[87] have, however, been found to accelerate the opening of the lactone ring of dehydroascorbic acid.

a. Peroxidase

The first of the enzymic mechanisms catalyzing the indirect oxidation of ascorbic acid to be described was the peroxide-peroxidase system.[88] Purified peroxidase preparations with H_2O_2 failed to catalyze the reaction and did so only after the addition of small amounts of plant juices.[88] The substances present in the plant juice which were responsible for restoring the activity were found to be phenolic compounds capable of quinone formation. Hence the reactions involved may be generally expressed as:

$$\text{Phenolic compound} + H_2O_2 \xrightarrow{\text{peroxidase}} \text{Quinone}$$

$$\text{Quinone} + \text{AA} \longrightarrow \text{DHA} + \text{phenolic compound}$$

All quinone-forming phenols are capable of acting as carriers in the reaction, though the most efficient were the naturally occurring *o*-dihydroxybenzopyrene pigments (flavones, flavonones, and flavonols).[89] Pseudoperox-

[83] R. W. Herbert, E. L. Hirst, G. V. Percival, R. Reynolds, and F. Smith, *J. Chem. Soc.* **52**, 1270 (1933).
[84] J. R. Penney and S. Zilva, *Biochem. J.* **37**, 39 (1943).
[85] E. R. Waygood, *Can. J. Research* **28**, 7 (1950).
[86] F. E. Huelin, *Australian J. Sci. Research* **2**, 346 (1949).
[87] L. W. Mapson and M. Ingram, *Biochem. J.* **48**, 551 (1951).
[88] A. Szent-Györgyi, *Biochem. J.* **22**, 1387 (1928).
[89] S. Huzak, *Hoppe-Seyler's Z. physiol. Chem.* **243**, 239 (1937).

idases, such as hemin or hemoglobin, have an action similar to that of peroxidase.[90]

b. Cytochrome c-Cytochrome Oxidase System

Cytochrome c will oxidize many mild reducing agents such as cystine, *p*-phenylenediamine, hydroquinone, and other phenols.[91] It is not surprising, therefore, that it will oxidize ascorbic acid. If, in addition, cytochrome oxidase is present, a cyclic oxidation system is established whereby ascorbic acid may be oxidized to completion, the oxidation being carried to the dehydroascorbic acid stage.[91] Cytochrome oxidase, in the absence of cytochrome c, will not oxidize ascorbic acid; hence the oxidation is an indirect one.[91, 92] The cytochrome system is widely distributed throughout animal tissues, and evidence is fast accumulating of its equally wide distribution and similar function in plant tissues.[93] Cytochrome oxidase is inhibited by HCN, H_2S, and azide, and in the dark by carbon monoxide,[92] the latter inhibition being reversed in light which causes dissociation of the carbon monoxide-enzyme complex. Such behavior is typical of an iron proteinate and is, so far as is known, not true for any other heavy metal enzyme.

c. Polyphenoloxidase

The identification of polyphenoloxidase in potatoes as a copper proteinate,[94] together with evidence from succeeding studies on previously reported oxidases,[95, 96] has indicated that copper associated with certain specific proteins will account for the oxidase activity previously designated as polyphenolase, potato oxidase, catechol oxidase, and tyrosinase. Polyphenolase converts *o*- and *p*-polyhydroxyphenols to the corresponding quinone. These oxidation products will, in turn, oxidize ascorbic acid to dehydroascorbic acid, and the system becomes a cyclic one until all the ascorbic acid is oxidized. The reaction may be expressed thus:

$$O_2 + \text{phenolic compound} \xrightarrow{\text{polyphenoloxidase}} \text{Quinone}$$

$$\text{AA} + \text{quinone} \longrightarrow \text{DHA} + \text{phenolic compound}$$

The most outstanding example of this type of reaction is the system in

[90] G. Bancroft and K. A. C. Elliott, *Biochem. J.* **28**, 1911 (1934).
[91] D. Keilin and E. F. Hartree, *Proc. Roy. Soc.* (*London*) **B125**, 171 (1938).
[92] E. Stotz, C. J. Harrer, M. O. Schultze, and C. G. King, *J. Biol. Chem.* **124**, 745 (1938).
[93] R. Hill and K. Bhagvat, *Nature* **143**, 726 (1939); K. Bhagvat and R. Hill, *New Phytologist* **50**, 112 (1950); P. B. Marsh and D. R. Goddard, *Am. J. Bot.* **26**, 724 (1939); D. R. Goddard, *ibid.* **31**, 270 (1944).
[94] F. Kubowitz, *Biochem. Z.* **292**, 221 (1937).
[95] F. R. Dalton and J. M. Nelson, *J. Am. Chem. Soc.* **60**, 3085 (1938).
[96] D. Keilin and T. Mann, *Nature* **143**, 23 (1939).

which catechol acts as carrier, large amounts of ascorbic acid being rapidly oxidized in the presence of quite small amounts of catechol.[97] Polyphenoloxidase is inhibited by cyanide, sulfide, and carbon monoxide,[98] the inhibition due to the latter not being reversed by light. The wide distribution of this type of enzyme in plants and fungi suggests that it may be an important terminal oxidase, and in potatoes it has been claimed that, as such, its activity may account for two-thirds of the total respiratory activity.[99] However, this viewpoint has recently been contested.[100] Many plants which do not possess ascorbic oxidase contain a high concentration of polyphenoloxidase in their tissues.

d. Laccase

This enzyme resembles polyphenoloxidase, since it catalyzes the oxidation of *o*- and *p*-phenolic derivatives to *o*- and *p*-quinones, but it differs in that it will not oxidize tyrosine or *p*-cresol.[96] Crude preparations of the enzyme oxidize ascorbic acid, but this activity decreases as the enzyme is purified; it can be restored by the addition of substances like phenylenediamine but not by catechol. The oxidation of ascorbic acid is therefore indirect. The enzyme is a copper proteinate containing 0.24% copper. An enzyme resembling laccase has been obtained from animal sources; it will oxidize catechol and hydroquinone, but not tyrosine or *p*-cresol;[101] its action on ascorbic acid has not been reported.

e. Ascorbic Oxidase

An enzyme catalyzing the oxidation of ascorbic acid by atmospheric oxygen was first described by Szent-Györgyi in 1931.[79, 102] Since these experiments, many workers have prepared the enzyme from a variety of plant tissues. Almost all authors agree that the enzyme is absent from mammalian tissues. The enzyme has been purified and has been obtained in crystalline form from the fruit of *Cucurbita*.[103] It is a blue or greenish-blue copper proteinate containing approximately 0.25% copper. It is the only enzyme known which catalyzes a direct oxidation of ascorbic acid by molecular oxygen to dehydroascorbic acid. Unlike the corresponding oxidation catalyzed by Cu^{++},[104] no H_2O_2 is found during the reaction.[105] Artificial copper

[97] D. Keilin and T. Mann, *Proc. Roy. Soc.* (*London*) **B125**, 187 (1938).
[98] D. Keilin, *Proc. Roy. Soc.* (*London*) **B104**, 206 (1929).
[99] J. G. Boswell and G. C. Whiting, *Ann. Bot.* **2**, 847 (1938).
[100] H. Levy and A. Schade, *Arch. Biochem.* **19**, 273 (1948); A. Schade and H. Levy *ibid.* **20**, 211 (1949).
[101] J. F. Cadden and L. V. Dill, *J. Biol. Chem.* **143**, 105 (1942).
[102] A. Szent-Györgyi, *Science* **72**, 125 (1930).
[103] T. Tadokoro and H. Takasugi, *J. Chem. Soc. Japan* **60**, 188 (1939).
[104] L. W. Mapson, *Biochem. J.* **39**, 228 (1945).

proteinates have been prepared which exhibit many of the properties of ascorbic oxidase. It would appear premature, however, to consider the natural enzyme simply as a complex of copper with a non-specific protein. Ascorbic oxidase oxidizes, besides L-ascorbic acid, certain analogs of ascorbic acid; D-araboascorbic acid, L-glucoascorbic acid, and L-galactoascorbic acid are equally oxidized by the enzyme.[106] Compounds related to ascorbic acid in which the oxygen bridge is on the right side of the carbon chain are directly oxidized by the enzyme at a much higher rate than are their enantiomorphs. Of these latter the six-membered series (D-ascorbic acid and D-araboascorbic acid) are oxidized more rapidly than the seven-membered series (D-glucoascorbic acid and D-galactoascorbic acid). Later work[107] has shown that other dienols (e.g., reductone and reductic acid) may be oxidized by the enzyme although at a much slower rate than the L-ascorbic acid series. The main specificity of the enzyme seems centered round the dienol grouping. The most noteworthy feature in the kinetics of ascorbic oxidase is the characteristic linear oxidation rates obtained,[108] which show no falling off in velocity until the substrate is completely oxidized. This implies a very low Michaelis constant, which has been determined to be of the order of 2×10^{-6} M.[109] The enzyme is extremely sensitive to cyanide; concentrations of 10^{-3} M will inhibit completely, and definite inhibitions may be observed with 10^{-5} M.[110]

2. Enzymic Systems Associated with the Reduction of Dehydroascorbic Acid

That ascorbic acid and dehydroascorbic acid are interconvertible in plant and animal tissue seems certain. In some fruits, notably apples, the proportion of dehydroascorbic acid to that of ascorbic acid is high, but as the fruit approaches maturity the concentration of dehydroascorbic acid decreases while that of ascorbic acid increases.[111] In germinating seeds it has also been found that the proportion of dehydroascorbic acid falls as the ascorbic acid increases.[112]

In most fresh plant or animal tissues, the concentration of dehydroascorbic acid is very small, compared to the concentration of ascorbic acid. It is, therefore, reasonable to assume that the enzymic systems associated

[105] H. G. Steinman and C. R. Dawson, *J. Am. Chem. Soc.* **64**, 1212 (1942).
[106] S. W. Johnson and S. S. Zilva, *Biochem. J.* **31**, 1366 (1937).
[107] G. A. Snow and S. S. Zilva *Biochem. J.* **32**, 1926 (1938).
[108] F. G. Hopkins and J. Morgan, *Biochem. J.* **30**, 1446 (1936).
[109] E. M. Crook, Ph.D. Dissertation, Cambridge, 1941.
[110] M. Srinivasan, *Biochem. J.* **30**, 2077 (1936).
[111] S. S. Zilva, F. Kidd, and C. West, *New Phytologist* **37**, 345 (1938).
[112] L. W. Mapson, Unpublished data.

with oxidation and reduction of ascorbic acid in the cell are poised at a level which is sufficient to maintain most of the ascorbic acid in the reduced form. This balance is normally maintained throughout the life of the cell. It may, however, be disturbed (1) by mechanical damage to the cell,[113] (2) by the action of narcotics, and (3) by the action of certain enzymic poisons.[64] Under such conditions, there is a rapid conversion of ascorbic acid to dehydroascorbic acid. The exact reason for these effects is not known. On the one hand it may be explained by the hypothesis that damage to the cell allows the oxidative enzyme systems to come into more effective contact with their substrates, resulting in a greatly increased rate of oxidation; a second explanation is that the enzymic systems concerned with the reduction of ascorbic acid are damaged to a greater extent than the oxidase systems. Whichever explanation is correct, it seems certain that in tissues reducing systems are present which maintain ascorbic acid in the reduced form. Thus both animal and plant tissues are capable of forming ascorbic acid from dehydroascorbic acid. Dehydroascorbic acid has been shown to possess antiscorbutic properties[114] and to form ascorbic acid on entry into plant cells.[64, 115]

Although the enzymic oxidation of ascorbic acid is relatively easy to demonstrate, the demonstration of enzymes or enzymic systems responsible for the reduction of dehydroascorbic acid presents a more difficult problem. The failure to discover any appropriate reducing system has in the past been the chief obstacle to accepting Szent-Györgyi's suggestion that in some species ascorbic acid might play the role of a respiratory catalyst.

Evidence of a reducing system in barley has, however, been found by James and his collaborators.[115] Having demonstrated the existence of an ascorbic oxidase in barley, these workers found that, out of several organic acids tried, three acids, namely glycollic, lactic, and tartaric, increased the oxygen consumption of barley saps in the presence of ascorbic acid. The increased oxygen consumption due to the presence of lactate was greater than with the other two acids. In the latter case, pyruvate was isolated as the oxidative product. The increased oxygen consumption due to these acids was not accompanied by any increased loss of ascorbic acid, and it appeared, therefore, that this effect was due to the oxidation of the organic acid, and not to that of ascorbic acid. They further demonstrated that barley sap increased the reduction of dyes such as methylene blue or 2,6-

[113] A. Szent-Györgyi, *J. Biol. Chem.* **90**, 385 (1931); W. Stone, *Biochem. J.* **31**, 508 (1937).

[114] K. I. Strakitskiĭ and B. A. Rubin, *Biokhimiya* **1**, 642 (1936).

[115] W. O. James and J. M. Cragg, *New Phytologist* **42**, 28 (1943); W. O. James, C. R. C. Heard, and G. M. James, *ibid.* **43**, 62 (1944).

dichlorophenolindophenol under anaerobic conditions when α-hydroxy acids were added. They concluded, therefore, that a mechanism for the reduction of dehydroascorbic acid exists in barley involving the participation of a dehydrogenase (or group of dehydrogenases) of α-hydroxy acids. They postulated that the following reactions may occur:

$$R\cdot CHOH\cdot COOH + DHA \rightarrow R\cdot CO\cdot COOH + AA \quad (1)$$

$$AA + O_2 \rightarrow DHA + H_2O \quad (2)$$

In further experiments it was shown that, with barley saps to which hexose diphosphate and ascorbic acid was added, an increased oxygen uptake occurred in excess of that caused by the addition of ascorbic acid alone; the oxygen uptake was still further increased by the addition of coenzyme I (diphosphopyridine nucleotide). The hexose diphosphate was broken down to phosphoglyceric acid, and it was shown that the reaction was stimulated by the addition of ascorbic acid. The course of hydrogen transport in these experiments is therefore believed to be triose phosphate → coenzyme I → ascorbic acid → O_2. How far coenzyme I was active in the former experiments was not and has not since been determined. The evidence in these studies is highly suggestive of a reducing mechanism, probably involving coenzyme I, for the reduction of dehydroascorbic acid. It is, however, only suggestive and not conclusive, for the authors did not demonstrate either with their lactate dehydrogenase or hexose diphosphate systems the direct reduction of dehydroascorbic acid to ascorbic acid.

Results of a similar character have been found in pea seeds and pea seedlings.[116] These tissues contain an active formic dehydrogenase which will reduce in the presence of coenzyme I dyes such as Nile blue; the reduced dye is in turn oxidized on the addition of dehydroascorbic acid. The oxygen consumption of such tissue is increased when formate is added and still further increased on the addition of ascorbic acid. Such evidence has been taken to indicate that hydrogen is transported via the coenzyme I-dehydroascorbic acid-ascorbic acid system. As in the work of James *et al.*, no direct demonstration of the reduction of dehydroascorbic acid in the presence of tissue extract, formate, or coenzyme I was made.

Further support for the hypothesis that any system producing dihydrocoenzyme I in cells may produce ascorbic acid from dehydroascorbic acid comes from recent work on wheat seedlings.[85] Cell-free extracts of wheat seedlings were found to contain a malic dehydrogenase-reducing coenzyme I as well as peroxidase and ascorbic oxidase systems. When to such extracts malic acid, coenzyme I, ascorbic acid, and a fixative for the oxalacetate formed in the reaction were added, the system absorbed oxygen in excess of that required for the complete oxidation of ascorbic acid. A similar

[116] D. C. Davison, *Proc. Linnean Soc. N.S. Wales* **74**, 37 (1949).

result was obtained if methylene blue replaced ascorbic acid. It was further found that in such a system, when the ascorbic acid was being oxidized, a pigment was formed in solution; the more rapid the rate of oxidation, the more rapid the formation of the pigment. This pigment was formed only when ascorbic acid was being oxidized and could not be produced in the absence of ascorbic acid by other oxidants such as oxygen or H_2O_2. On the available evidence, the suggestion was made that the pigment exists in a colored oxidized state and a colorless reduced state, and that the leuco pigment reacts only with dehydroascorbic acid as follows:

$$\text{Leuco pigment} + \text{dehydroascorbic acid} = \text{Pigment} + \text{ascorbic acid}$$

Since both oxygen and ascorbic acid were found to be necessary for the development of the pigment, it was concluded that dehydroascorbic acid was directly responsible and, moreover, that the pigment as a carrier of hydrogen must be positioned between coenzyme I and ascorbic acid for, if it were a terminal carrier, oxygen and not dehydroascorbic acid would be required for its development.

The nature of this pigment was not identified, but the suggestion was made that it might be identical with or similar to either Palladine's respiratory chromogens,[117] Robinson's leucoanthocyanins,[118] the respiratory pigment Hermidin extracted from *Mercurialis*,[119] Szent-Györgyi's vitamin P[120] or the oxidative-reduction enzyme reported by Wawra and Webb.[121] On the basis of their work, the author has proposed the following scheme for the system:

$$\text{Malate} \xrightarrow{\text{malic dehydrogenase}} \underset{\text{Coenzyme I } H_2}{\overset{\text{Coenzyme I}}{\rightleftarrows}} \rightarrow \underset{\text{Flavin}}{\overset{\text{Flavin } H_2}{\rightleftarrows}} \rightarrow \underset{\text{Pigment}}{\overset{\text{Leuco pigment}}{\rightleftarrows}} \rightarrow \underset{\text{AA}}{\overset{\text{DHA}}{\rightleftarrows}} \rightarrow \underset{\text{Ascorbic oxidase}}{O_2}$$

Further evidence of a link between coenzyme I and ascorbic acid has recently been reported.[122] An enzyme preparation has been obtained from green peas which catalyzes the oxidation of reduced coenzyme I by oxygen in the presence of either methylene blue or ascorbic acid. This reaction was found to be insensitive to cyanide. The enzyme preparation catalyzed the oxidation of reduced coenzyme II with methylene blue but not with ascor-

[117] W. Palladin, *Biochem. Z.* **27**, 442 (1910).
[118] G. M. Robinson and R. Robinson, *J. Chem. Soc.* **1935**, 744.
[119] P. Haas and T. G. Hill, *Ann. Bot.* **39**, 861 (1925).
[120] A. Bentsath, St. Rusznak, and A. Szent-Györgyi, *Nature* **139**, 326 (1937).
[121] C. Z. Wawra and J. L. Webb, *Science* **96**, 302 (1942).
[122] B. Matthews, *J. Biol. Chem.* **189**, 695 (1951).

bic acid. The preparation thus contained a diaphorase I and II similar in character to those reported in peas. The hydrogen acceptor in the reaction was not dehydroascorbic acid, prepared by the oxidation of ascorbic acid with iodine. The suggestion was made that the hydrogen acceptor is a semiquinone intermediate (monodehydroascorbic acid) of ascorbic acid. If this suggestion is correct, it may well be the reason why the present writer was unable to demonstrate the reduction of dehydroascorbic acid by either coenzyme I-specific formate or ethanol dehydrogenase enzymes from peas with added coenzyme I.

a. Role of Glutathione (GSH)

There has been much work indicating the close association between GSH and ascorbic acid in plant and animal tissue.[123] In germinating seeds and sprouting potato tubers, both these substances appear at the same time. When potato tubers are cut or subjected to ethylene chlorohydrin,[124] their content of both GSH and ascorbic acid increases, and the formation of both substances is abolished under anaerobic conditions. In animal tissue, Szent-Györgyi (1928) first showed[125] that in minced rat kidney dehydroascorbic acid could be reduced and that this reduction could be effected by GSH in buffer solutions. This work was extended to liver tissue in which it was found that GSH protected ascorbic acid from oxidation.[108] The protective effect of GSH on the oxidation of ascorbic acid has been observed by several workers, but the explanation of this has been ascribed to different causes. The fact that GSH combines readily with copper and that ascorbic acid may be protected from oxidation catalyzed by copper in the presence of sulfhydryl compounds is believed by some workers to be one explanation for the stabilizing effect of GSH on ascorbic acid.

That this is not the sole explanation is shown by the fact that GSH will reduce dehydroascorbic acid (DHA) in solution without addition of added catalysts.[126] The rate of this uncatalyzed reaction is, however, too slow to be of much consequence either in maintaining ascorbic acid in the reduced form in tissues, or as part of a respiratory cycle. The "half-time" period for the reduction of DHA by GSH at physiological temperatures and pH and in concentrations usually found *in vivo* is of the order of 15 minutes;[109] on the other hand, under the same conditions the conversion of DHA to 2,3-diketogulonic acid itself has a half-life of only 2 minutes.[127]

Pfankuch[128] was the first to describe the catalytic reduction of DHA by

[123] L. B. Pett, *Biochem. J.* **30,** 1228 (1936); F. G. Hopkins and E. J. Morgan, *Nature* **152,** 288 (1943).

[124] J. Guthrie, *Contribs. Boyce Thompson Inst.* **9,** 17 (1937).

[125] A. Szent-Györgyi, *Biochem. J.* **22,** 1387 (1928).

[126] E. M. Crook and F. G. Hopkins, *Biochem. J.* **32,** 1356 (1938).

[127] M. Ball, *J. Biol. Chem.* **118,** 219 (1937).

sulfhydryl compounds. He showed the presence in potato juice of an enzyme which catalyzed the reduction of DHA by cysteine. The catalyst responsible was found to be a heat-labile substance and to be precipitated by protein reagents. This work was extended notably by Hopkins and his collaborators,[129] who demonstrated the presence of an enzyme, dehydroascorbic reductase, which catalyzed the reduction of dehydroascorbic acid by GSH in accordance with the following reaction:

$$2GSH + DHA \rightleftharpoons GSSG + AA$$

The enzyme was prepared from cauliflower and separated from ascorbic oxidase. It had the properties of an enzyme in that it was thermolabile, was nondiffusible, and was precipitated by $(NH_4)_2SO_4$, and its activity was affected by pH in a manner characteristic of enzymes. The enzyme catalyzed the reduction of DHA by cysteine, thiolactic acid, or GSH, although the latter compound was twice as effective as cysteine and four times as effective as thiolactic acid in equimolar concentration. The distribution of the enzyme was investigated and was found to be present in twenty-two of the thirty species examined, the most active sources being cauliflower and broad beans. So far no enzyme of this type has been found in animal tissues.

Other workers[130] were unable to repeat the observations of Hopkins and his collaborators for reasons which are obscure. However, a re-examination of the earlier work produced confirmatory evidence for the existence of this enzyme. The presence of a dehydroascorbic reductase in leguminous seeds was also found by Volinar and Sansom, although their work suggested that it was not glutathione but another system that was oxidized by dehydroascorbic acid. The present writer (unpublished experiments) has been able to substantiate the work of Hopkins and his coworkers in showing the presence of an enzyme catalyzing the reaction between GSH and DHA in cress seedlings, potatoes, and peas.

The possibility that the dehydroascorbic acid-glutathione system is one which may form a hydrogen transfer system similar to that of the cytochrome system was suggested by Crook (1941).[129] The system was visualized as acting as follows:

$$H \xrightarrow[\text{substrates}]{\text{plant}} -S-S- \xrightarrow[\text{reductase}]{\text{dehydroascorbic}} DHA \xrightarrow[\text{oxidase}]{\text{ascorbic}} \text{Atmospheric } O_2$$

[128] F. Pfankuch, *Naturwissenschaften* **22**, 821 (1934).

[129] F. G. Hopkins and E. J. Morgan, *Biochem. J.* **30**, 1446 (1936); E. M. Crook and F. G. Hopkins, *Biochem. J.* **32**, 1356 (1938); E. M. Crook, *Biochem. J.* **35**, 226 (1941); E. M. Crook and E. J. Morgan, *Biochem. J.* **38**, 10 (1943); E. F. Kohman and N. H. Sanborn, *Ind. Eng. Chem.* **29**, 189, 1195 (1937).

[130] A. Fujita and I. Numata, *Biochem. Z.* **299**, 249 (1938); Z. I. Kertesy, *Biochem. J.* **32**, 621 (1938).

This suggestion was criticized by Barron[131] on the grounds that there is very little GSH in plants and a large amount of ascorbic acid. The answer to this is that the concentrations of both GSH and ascorbic acid used in Hopkin's experiments were well within the physiological ranges known to occur in plants, and neither were the ratios of GSH and ascorbic acid unphysiological, since GSH and ascorbic acid occur in a molar ratio of 2:1 in one plant at least (potato tubers).

Evidence has been accumulating that both plant and animal tissues possess the power to reduce —S—S— compounds to SH compounds. In germinating seeds sulfhydryl groups appear rapidly after hydration of the seeds,[132] and the sulfhydryl compounds produced have been shown to be mainly glutathione.[133] Oxidized glutathione, when added to powdered pea seeds, was rapidly converted to the reduced form. With animal tissues GSSG may be reduced to GSH in the presence of glucose dehydrogenase and an activator prepared from liver tissue. With extracts of blood and yeast, GSSG was reduced in the presence of hexose monophosphate and extracts containing coenzyme II.[134]

Bukin[135] related the glutathione-ascorbic acid systems to coenzyme I on the evidence that oxidized glutathione may be reduced by dihydrocoenzyme I in a simple non-enzymic reaction. The rate of enzymic oxidation of glutathione has also been found to be increased by the presence of coenzyme I in kidney homogenates, and these reactions are stimulated by the presence of ascorbic acid both in the presence and absence of cytochrome c.[136]

The claim that dihydrocoenzyme I will reduce oxidized glutathione has not, however, been substantiated by other workers, either as a non-enzymic reaction or by systems containing dehydrogenase enzymes and substrates with added coenzyme I and oxidized glutathione.[137, 138] It seems probable that earlier workers may have had coenzyme II as an impurity in their coenzyme I preparations. Until recently, however, the evidence for the cooperation of glutathione with coenzyme-linked reducing systems has been contradictory and based in plants on the premise of a non-enzymic reaction. Recent work,[137, 138] however, has demonstrated the existence in many plant

[131] E. S. G. Barron, *Cold Spring Harbor Symposia Quant. Biol.* **7**, 145 (1939).

[132] M. J. Firket and M. Comhaire, *Bull. acad. roy. méd. Belg.* **9**, 93 (1929); R. Vivario and J. LeClaux, *Arch. Intern. Physiol.* **32**, 1 (1930).

[133] F. G. Hopkins and E. J. Morgan, *Nature* **152**, 288 (1943).

[134] P. J. G. Mann, *Biochem. J.* **26**, 785 (1932).

[135] V. N. Bukin, *Biokhimiya* **18**, 60 (1943).

[136] E. Stotz, C. J. Harrer, M. O. Schultze, and C. G. King, *J. Biol. Chem.* **122**, 407 (1938).

[137] L. W. Mapson and D. R. Goddard, *Nature* **167**, 975 (1951); L. W. Mapson and D. R. Goddard, *Biochem. J.* (In press.)

[138] E. E. Conn and B. Vennesland, *Nature* **167**, 976 (1951).

tissues of an enzyme catalyzing the reduction of oxidized glutathione by dehydrocoenzyme II. This enzyme, glutathione reductase, has been shown to catalyze the reaction

$$GSSG + Co\ II{\cdot}2H \rightarrow 2GSH + Co\ II$$

The reverse reaction could not be shown, even in the presence of excess of GSH, indicating that the equilibrium of the reaction is in favor of the formation of reduced glutathione. The enzyme appears to be highly specific for glutathione: it will not catalyze the reduction of cystine, homocystine, α-glutamylcystine, or aspartathione. It is also specific for coenzyme II. There is no reaction between dihydrocoenzyme I and oxidized glutathione.

The possibility of a hydrogen transfer system involving coenzyme II glutathione and ascorbic acid is at once apparent. In pea seeds the transfer of hydrogen from substrates such as isocitrate or malic to dehydroascorbic and coenzyme II and GSSG has been shown to occur in accordance with the following reactions:[137]

$$Co\ II + \text{isocitrate} \xrightarrow[\text{dehydrogenase}]{\text{isocitric}} Co\ II\ H_2 + \text{oxalosuccinate}$$

$$\text{Oxalosuccinate} \xrightarrow{Mn} \alpha\text{-Ketoglutarate} + CO_2$$

$$Co\ II\ H_2 + GSSG \xrightarrow[\text{reductase}]{\text{glutathione}} 2GSH + Co\ II$$

$$GSH + DHA \xrightarrow[\text{reductase}]{\text{dehydroascorbic acid}} AA + GSSG$$

We do not know at present how important such a system is in the general respiratory activity of plant or animal cells. As far as our present information goes, the enzyme system, like that of cytochrome and the other terminal oxidases, will be inhibited by cyanide. Direct evidence that there is a connection between the state of oxidation of GSH and that of ascorbic acid has recently been shown in potato tubers. When these are subjected to atmospheres of pure oxygen there is, after a period in which no apparent change occurs, first a fall in the GSH content of the tissue, followed by a fall in the concentration of ascorbic acid and a rise in that of dehydroascorbic acid.[139]

b. Reduction of Dehydroascorbic Acid by Bacteria

Several workers have reported that certain bacteria can reduce dehydroascorbic acid to ascorbic acid.[140] Strains of *Escherichia coli* and of *Aerobacter*

[139] J. Barker and L. W. Mapson, *New Phytologist* (In press.)

[140] I. C. Gunsalus and D. B. Hand, *J. Biol. Chem.* **141**, 853 (1941); A. P. Stewart and P. F. Sharp, *Ind. Eng. Chem. Anal. Ed.* **17**, 373 (1945); L. W. Mapson and M. Ingram, *Biochem. J.* **48**, 551 (1951).

aerogenes, *Salmonella pullorum*, and *Salmonella enteritidis* were found to be active. Further examination has shown that only *Staphylococci* and members of the Enterobacteriaceae[141] bring about the reduction. With intact cells, various substrates may serve as hydrogen donators; these include glucose, lactate, formate, succinate, malate, alcohol, and hydrogen. Cell-free dehydrogenase enzymes prepared from *E. coli* were ineffective, although such preparations were active in reducing methylene blue. Some other factor[142] (possibly a carrier) is involved in addition to the dehydrogenases supplying hydrogen. This unknown factor is not identical with or replaceable by methylene blue, pyocyanin, glutathione, coenzyme II, yeast extract, or boiled or unboiled yeast. Besides being able to reduce dehydroascorbic acid, the *Staphylococci* and the Enterobacteriaceae are peculiar in containing cytochrome b_1. Spectroscopic observations have indicated that cytochrome b_1 is oxidized (though incompletely) when dehydroascorbic acid is added. It has been suggested, therefore, that cytochrome b_1 may be an essential component of the reducing system.

Although no bacterium not containing cytochrome b_1 has so far been found which will reduce DHA, some bacteria have been examined which contain cytochrome b_1 but which will not reduce dehydroascorbic acid. An additional factor besides cytochrome b_1 appears, therefore, to be involved. This may be a dehydroascorbic acid reductase, since leuco-Nile blue is reoxidized by dehydroascorbic acid more strongly in the presence of cells of a strain which can effect this reduction than with those of a strain which cannot.

3. Ascorbic Acid and the Metabolism of Tyrosine

When 0.5 g. or more of L-tyrosine is administered to scorbutic guinea pigs, homogentisic, *p*-hydroxyphenylpyruvic, and *p*-hydroxyphenyllactic acids are excreted in the urine.[143] These compounds are not formed if the tyrosine is administered with L-ascorbic acid. Similar observations made in infants[144] have led to the suggestion that L-ascorbic acid participates directly or indirectly in protein metabolism.

These results have generally been confirmed by other workers,[145] but it has been pointed out that very high doses of L-tyrosine must be consumed by guinea pigs on a scorbutic diet before the failure to rupture the benzene

[141] B. P. Eddy, *Biochem. J.* (In press.)

[142] B. P. Eddy, M. Ingram, and L. W. Mapson, *Proc. Soc. Gen. Microbiol.* (1951); *Biochem. J.* (In press.)

[143] R. R. Sealock and H. E. Silberstein, *Science* **90, 517** (1939); *J. Biol. Chem.* **135,** 251 (1940).

[144] S. Z. Levine, E. Marples, and H. H. Gordon, *Science* **90,** 620 (1939); *J. Clin. Invest.* **20,** 209 (1941).

[145] H. A. Painter and S. S. Zilva, *Biochem. J.* **41,** 511 (1947).

ring in a part of the ingested compound can be observed. Secondly, this biochemical abnormality becomes effective very soon after the L-ascorbic acid has been removed from the diet, the maximum excretion of hydroxyphenyl compounds occurring as early as 24 to 48 hours after the animals were deprived of the vitamin. In other words this abnormality appears even when the tissues of the guinea pig are still saturated with L-ascorbic acid. The phenomenon is also seen in animals which are capable of synthesizing their own ascorbic acid if high doses of tyrosine are given.[146] Abnormal excretion of intermediate products may therefore occur even in the normal animal. The necessity for the presence of an excess of L-ascorbic acid in order that all the tyrosine shall be completely metabolized is no indication of the existence of a connection between the normal function of L-ascorbic acid and the metabolism of aromatic or of other amino acids. The apparent interaction *in vivo* between L-ascorbic acid and L-tyrosine suggests rather a physiological response to an unusual situation. Subsequent work has shown that the production of hydroxyphenyl compounds from tyrosine may occur in the intestinal tract of scorbutic guinea pigs and that the accumulation of these compounds is inhibited by the presence of L-ascorbic acid.[147] If bactericidal agents are fed in combination with L-ascorbic acid, there is an accumulation of hydroxyphenyl compounds in the intestine. The suggestion is made that L-tyrosine is normally quickly destroyed by microorganisms and that the rate of the destruction can be slowed down either by administering bactericidal agents or by withholding L-ascorbic acid. The presence of L-ascorbic acid may favor those microorganisms capable of attacking phenolic groups. The vitamin may even act in the chain of reactions concerned in the degradation both in bacterial and animal cells. This appears probable from work on liver slices from normal and scorbutic guinea pigs.[148] The oxygen uptake of liver slices from normal animals is increased on the addition of tyrosine in an amount equivalent to one atom of oxygen per mole of tyrosine. This increased oxygen uptake was not observed, however, in liver slices from scorbutic animals, unless ascorbic acid was also added. The fact that both tyrosine and phenylalanine are converted to the aromatic keto acids, even in the scorbutic animal, suggests that oxidative deamination proceeds in the absence of ascorbic acid, but that the further oxidation of the aromatic keto acids is dependent on the presence of ascorbic acid. D-Glucoascorbic acid, which is biologically inactive, also accelerates these reactions to the same

[146] Y. Kotake, G. Matsuoka, and M. O. Kagawa, *Hoppe-Seyler's Z. physiol. Chem.* **122**, 166 (1922).

[147] H. A. Painter and S. S. Zilva, *Biochem. J.* **43**, 398 (1948).

[148] T. H. Lan and R. R. Sealock, *J. Biol. Chem.* **155**, 483 (1945); R. R. Sealock and T. H. Lan, *J. Biol. Chem.* **167**, 689 (1947).

extent as an equivalent amount of L-ascorbic acid.[149] This indicates that the chemical behavior of L-ascorbic acid in these reactions is not one of its essential roles in metabolism. The suggestion has been made that these substances accelerate the oxidation of the aromatic keto acids by virtue of their effect on the oxidative reduction potential of the system.

4. Action of Ascorbic Acid on Enzymes

Ascorbic acid has been found to have an effect on a number of enzymes. In some cases the effect observed has been one of activation, e.g., arginase,[150] papain,[151] β-amylase[152] from liver and pancreas, liver esterase, catalase,[153] and cathepsin;[150] in others inhibitory effects have been described, e.g., urease[154] and β-amylase of plants.[152] Several workers have attempted to demonstrate the effect of ascorbic acid by comparing the enzymic activity of extracts from tissues of normal and scorbutic animals. The blood esterase of scorbutic guinea pigs was found to be lower than in normal animals[155] and to be increased with the administration of ascorbic acid.[156] Similar results were observed in deficient guinea pigs for other enzymes, notably liver esterase, succinic dehydrogenase, and cytochrome oxidase.[157, 158] The lowered enzymic activity of tissue extracts from deficient animals does not necessarily indicate, however, that the lowered activity observed is directly due to an absence of the vitamin; it may well be caused by inanition accompanying the deficient state.

In the case of liver esterase the claim was made that the enzyme consists of a protein (apoenzyme) combined with ascorbic acid (coenzyme), when it was found that liver esterase loses its activity on dialysis with dilute HCl[156] but can be reactivated by the addition of ascorbic acid. These results were not confirmed by other workers.[159]

Many enzymes have been shown to depend for their activity on the integrity of an —SH group in the molecule, and the activating effect of ascorbic acid has been suggested as being due to the protection of such —SH groups from oxidation. However, it has been shown that the activity of one such enzyme, urease, is inhibited by ascorbic acid although this

[149] H. A. Painter and S. S. Zilva, *Biochem. J.* **46**, 542 (1950).
[150] H. Tauber, *Ergeb. Enzymforsch.* **4**, 42 (1935).
[151] E. Maschmann, *Hoppe-Seyler's Z. physiol. Chem.* **228**, 141 (1934).
[152] A. Purr, *Biochem. J.* **28**, 1141 (1934).
[153] F. Lenthardt and F. Koller, *Helv. Chim. Acta* **17**, 1030 (1934); C. A. Badimond, *Compt. rend. soc. biol.* **125**, 283 (1937).
[154] L. A. Elson, *Nature* **152**, 49 (1943).
[155] A. Palladin and P. Normark, *Biochem. Z.* **152**, 420 (1924).
[156] J. Moster, *Klin. Wochschr.* **15**, 1558 (1936).
[157] W. von Pantschenko-Jurewicz and H. Krant, *Biochem. Z.* **285**, 407 (1936).
[158] C. J. Harrer and C. G. King, *J. Biol. Chem.* **138**, 111 (1941).
[159] J. H. Quastel, *Nature* **152**, 215 (1943).

inhibition was eliminated in the presence of cysteine.[160] A suggestion that dehydroascorbic acid was the agent responsible for reacting with the —SH group of the enzyme[159] and that the protective effect of thiol compounds was due to the reduction of dehydroascorbic acid was shown to be untenable when it was shown that dehydroascorbic acid did not inactivate urease.[161] In further studies[160] it was found that ascorbic acid itself does not inhibit urease activity. In the presence of Cu^{++}, however, it does so by effecting the reduction of $Cu^{++} \rightarrow Cu^{+}$, which latter ions have a much higher affinity for —SH groups than the former. With mercury salts the reverse effect of ascorbic acid was observed, namely an activation of urease activity. This was correlated with the fact that enzymic activity was reduced more by Hg^{++} than by Hg^{+} salts. The action of ascorbic acid on urease could therefore be explained in terms of its reducing action on metallic ions present in solution. The inhibiting action of ascorbic acid on plant β-amylase is probably explicable on a similar basis, since it has been shown that the inhibition was increased in the presence of small amounts of copper salts.[152]

It is of interest to note that the activating effect of ascorbic acid on papain occurs only if ferrous ions are present; otherwise the effect of the vitamin is depressant.[162] There is evidence suggesting that the ascorbic acid-iron complex activates by first reducing dithio compounds associated with the enzyme, and that these thiol compounds in turn activate the enzyme.[163] Similar reactions may be involved in the activation of arginase by ascorbic acid-iron complex.[164]

The action of ascorbic acid on many enzymes appears to be conditioned by other substances, notably metallic ions, present in reaction mixtures; certainly where its action has been critically examined, this has been found to be so. Whether ascorbic acid *in vivo* has any regulatory influence on these enzymes is uncertain. Still more improbable is the view that the action of ascorbic acid constitutes one of its essential roles in the living cell, since in the case of urease it has been shown that other dienols which are biologically inactive react similarly.[160]

5. Ascorbic Acid and Phosphatase

As early as 1932 it was observed that the alkaline phosphatase activity of the plasma of infants and young children suffering from scurvy was low,[165]

[160] L. W. Mapson, *Biochem. J.* **40**, 240 (1946).

[161] K. Giri and P. Seshagiri Rao, *Nature* **153**, 253 (1944).

[162] L. Hellerman and M. Perkins, *J. Biol. Chem.* **107**, 241 (1934); E. Maschmann and E. Helmert, *Hoppe-Seyler's Z. physiol. Chem.* **224**, 56 (1934).

[163] A. Purr, *Biochem. J.* **29**, 513 (1935).

[164] A. Purr, *Biochem. J.* **27**, 1703 (1933); A. Purr and L. Weil, *Biochem. J.* **28**, 740 (1934).

[165] J. Smith and M. Maizels, *Arch. Disease Childhood* **1**, 149 (1932); J. Smith, *ibid.* **8**, 215 (1933).

and that enzymic activity was restored to normal on treatment with ascorbic acid. These results were substantiated for guinea pigs by several workers.[166-169] Scurvy is not the only disease, however, in which a decrease in the phosphatase of the blood occurs; diseases in which there is a stoppage of growth accompanied by loss of weight produce similar results.[165] How far the reduction of phosphatase activity is related specifically to a deficiency of L-ascorbic acid remains in doubt. Attempts to exclude the effect of inanition were made with guinea pigs by means of the paired-feeding technique,[167] but even under these conditions the scorbutic animals lost more weight than their paired controls, and the greater decrease in the phosphatase activity of the tissue of the scorbutic animals may have been due to this cause. More recently[170] a further attempt has been made to differentiate between inanition and cessation of growth on the one hand and the effect of L-ascorbic acid on the other on the phosphatase activity of both bone and serum. Evidence was obtained indicating that the fall in the phosphatase content of the serum and in the zone of provisional calcification of the costochondral junctions and the tibiae, observed during the development of scurvy, is caused by the scorbutic condition, although in the later stages the fall in weight may be a contributary factor.

That L-ascorbic acid *per se* does not regulate the phosphatase content of the tissue is seen from the fact that 18 hours after the intraperitoneal injection of sufficient of the vitamin to saturate the tissues no rise in the phosphatase activity of the tissues of scorbutic animals could be observed; only after 2 days when the therapeutic action of the vitamin was well in progress was the rise of the enzymic activity noted. Ascorbic acid does not, therefore, appear to act directly on the enzyme, but rather indirectly through restoring the scorbutic condition of the tissues to normal. Our knowledge at present is not sufficient to indicate whether the phosphatase activity characteristic of the scorbutic state results from an interference in the synthesis of the enzyme or whether it is due to a transference of the enzyme from some tissues to other tissues more affected by the deficiency. The serum phosphatase is the first of the phosphatases to decrease during the development of scurvy and the last to reach its normal level after cure, which suggests that it may fulfill some protective function. The fall in the phosphatase of bone may be due to a disturbance in the function of the osteoblasts caused by local scorbutic lesions. It is possible, therefore, that the low phosphatase content of bone may be due to poor synthesis, and

[166] G. Soz, C. Cattaneo, and M. C. Gabbrielli, *Enzymologia* **3**, 29 (1937).
[167] E. N. Todhunter and W. Brewer, *Am. J. Physiol.* **130**, 310 (1940).
[168] H. Schwachman and B. S. Gould, *J. Nutrition* **23**, 271 (1942).
[169] B. S. Gould and H. Schwachman, *J. Biol. Chem.* **151**, 439 (1943).
[170] H. R. Perkins and S. S. Zilva, *Biochem. J.*, **47**, 306 (1950).

the low content of serum to a redistribution of the enzyme between blood and other tissues.

6. Ascorbic Acid and Carbohydrate Metabolism

There have been many reports of some connection between ascorbic acid and the metabolism of carbohydrate. The capacity for metabolizing glucose is lowered in the scorbutic guinea pig,[171] and these animals show a diabetic type of response to glucose tolerance tests. It has also been reported that scorbutic guinea pigs show lower liver glycogen, glycosuria, and a reduced insulin content of the pancreas compared with normal animals.[172] The observation that dehydroascorbic acid has a diabetogenic effect in rats[173] is in line with these earlier observations. Furthermore, suggestive evidence that the synthesis of glycogen may be connected with ascorbic acid has been obtained[174] in experiments showing that the increase in liver glycogen which occurs in normal fasting guinea pigs following a gluconeogenic stimulus by the administration of adrenal cortical extract is not observed in the scorbutic animal. Experiments such as these seem to establish a difference in glycogen metabolism between scorbutic and normal animals, but more work is needed to ascertain the specificity of this action of ascorbic acid, and still more to work out the biochemical systems involved.

7. Ascorbic Acid and the Synthesis of the *Leuconostoc citrovorum* Factor

The observation that a factor was necessary for the growth of the bacterial strain *Leuconostoc citrovorum*[175] and that the growth factor was related to folic acid was followed by the further observation that liver slices from folic acid-deficient rats incubated with ascorbic acid alone approximately doubled the content of the citrovorum factor in the tissue. Still larger increases of the growth stimulant were observed if both folic acid and ascorbic acid were added to the liver slices. The conclusion from the results of these experiments was that folic acid is enzymatically converted into the citrovorum factor and that this conversion is aided by the presence of ascorbic acid.[175, 176]

The finding that ascorbic acid enhances the conversion of folic acid to citrovorum factor is of great interest since, if the conversion is essential for

[171] A. Sigal and C. G. King, *J. Biol. Chem.* **116**, 489 (1936); *J. Pharmacol. Exptl. Therap.* **59**, 468 (1937); **61**, 1 (1937).

[172] S. Banerjee, *Ann. Biochem. and Exptl. Med. (India)* **3**, 157 (1943); **4**, 33 (1944).

[173] J. W. Patterson, *Endocrinology* **45**, 344 (1949).

[174] R. W. McKee, T. S. Cobbey, and Q. M. Geiman, *Endocrinology* **45**, 21 (1949).

[175] H. E. Sauberlich and C. A. Baumann, *J. Biol. Chem.* **176**, 165 (1948); **181**, 871 (1949).

[176] E. E. Snell, E. Kitay, and W. S. McNutt, *J. Biol. Chem.* **173**, 473 (1948).

the biological action of folic acid, it provides a possible explanation of the previously reported relationships between folic acid and ascorbic acid.[177,178]

8. Ascorbic Acid and Formation of Intercellular Substances and Collagen

Another well-founded function of ascorbic acid is its participation in the formation of intercellular substances of cartilage, dentine, and bone, and the collagen of fibrous tissues. The pathological symptoms of the scorbutic state in which there is a disturbance of the metabolism which affects the bones and teeth, and hemorrhagic lesions in other tissues, appear to be associated with the basic function of ascorbic acid. The biochemical explanation of these pathological changes is still unknown and is therefore outside the scope of this review. The marked decrease in the phosphatase activity of scorbutic tissue may, however, be a contributory factor.

V. Estimation

MAMIE OLLIVER

A. BIOLOGICAL METHODS

The biological method for the estimation of vitamin C is specific for antiscorbutic activity and, as such, can be accepted as the final standard of reference in cases where it is suspected that the accuracy of the chemical or physical procedures might be affected by the presence of interfering substances. The biological test will measure the total amount of vitamin C present, i.e., both in the reduced form of ascorbic acid itself and also in the reversibly oxidized form of dehydroascorbic acid (the latter, however, generally occurring in natural products in no more than relatively small traces). The applicability of the biological method may be limited only if the potency of the test material is too low for it to be measured accurately or if the sample is poorly absorbed.

Rats cannot be used as test animals for ascorbic acid assay owing to their ability to synthesize the vitamin, but guinea pigs have proved satisfactory. A method based upon the determination of the minimum amount of sample needed to prevent signs of scurvy in the guinea pig was originally devised by Sherman and his coworkers.[1,2] The animals were fed on a basal diet

[177] C. A. Nichol and A. D. Welch, *Proc. Soc. Exptl. Biol. Med.* **74**, 52 (1950).
[178] *Nutrition Rev.* **7**, 49 (1949).
[1] H. C. Sherman, V. K. LaMer, and H. L. Campbell, *J. Am. Chem. Soc.* **44**, 165 (1922).
[2] H. C. Sherman and S. L. Smith, The Vitamins, 2nd ed. American Chemical Society, Monograph Series, Chemical Catalog Co., New York, 1931.

containing all known nutrients except ascorbic acid and supplemented with graded amounts of the test sample. One group was kept as negative controls. At the end of 6 to 10 weeks, the degree of protection against scurvy was determined by signs, survival period, and autopsy findings. The amount of test material just sufficient to prevent scurvy was taken as equivalent to one Sherman unit or 0.5 to 0.6 mg. of ascorbic acid.

Many modified procedures were subsequently suggested, and of these the most frequently used are the preventive dental histology method and the curative growth method. The dental histology method arose from the work of Höjer,[3] who described ten stages in histological changes in the teeth during the development of scurvy. These stages were based on the amount of disorganization of the odontoblasts, the width, irregularity and structure of the dentine, and the degree of calcification of the predentine. Key and Elphick[4] subsequently defined four arbitrary stages of deficiency, and the degree of protection is more usually assessed by reference to these standards. The general procedure is to place standardized guinea pigs for a few days on a basal scurvy-producing diet, supplemented with 15 g. of cabbage daily, until the weight is between 250 and 300 g. The animals are then divided into groups and for a period of 2 weeks are given the basal diet, supplemented with graded levels of the test substance in some groups and of ascorbic acid in others. Another group on the basal ration alone serves as a negative control. The animals are then killed, the lower jaws removed, and sections made of the decalcified incisors. The degree of protection is assessed by microscopic examination of the sections for the histological changes described above,[5] and by comparison of the results of the standard ascorbic acid with the sample doses.

In the simple curative method, use is made of the fact that, if male guinea pigs are placed on a basal scurvy-producing diet, a decline in weight begins on about the eleventh day.[6] The animals, each weighing exactly 300 g., are therefore given this diet for 10 days and then, before any appreciable loss in weight has yet occurred, are placed on graded doses of the test sample and of ascorbic acid, a further group of the guinea pigs being kept as negative controls. Changes of body weight are recorded daily over a further period of 20 to 30 days, and the biological values of the sample are calculated from a comparison of the resultant dose-response curves. The advantage of this over the previous method from the point of view of time and simplicity is self-evident, and, although theoretically the specificity might be regarded as less, this is not found in practice.

[3] J. A. Höjer, *Brit. J. Exptl. Pathol.* **7**, 356 (1926).

[4] K. M. Key and G. K. Elphick, *Biochem. J.* **25**, 888 (1931).

[5] K. H. Coward, Biological Standardization of the Vitamins, 2nd ed., p. 93. Baillière, Tindall and Cox, London, 1947.

[6] L. J. Harris and M. Olliver, *Biochem. J.* **36**, 155 (1942).

A further quantitative bioassay method has been worked out, based upon the observation that the level of serum "alkaline" phosphatase, i.e., the enzyme with an optimum activity at pH 8.6 to 9.0, falls considerably in scurvy and increases again when ascorbic acid is administered.[7] A curative rather than preventive procedure is recommended, owing to the more consistent behavior of the animals. Consequently, the procedure adopted is first to reduce the level of serum "alkaline" phosphatase in the experimental animals to about 4 to 5 units, and then to administer doses of the test sample at varying ascorbic acid levels. The serum phosphatase is determined at various intervals from 3 to 15 days, and the ascorbic acid content is determined on the basis that 0.225 mg. of ascorbic acid per day is the critical dose for a 5-day response, and 0.20 mg. for a 10-day response.

Apart from the precautions which normally have to be observed in the biological assay of any vitamin, special care has to be taken when estimating the ascorbic acid content of fresh fruit and vegetables, in order to ensure uniformity of the sample dosed. For instance, a constant source of supply of the material under test is necessary in order to minimize the effect of varietal, soil, and other conditions on the vitamin (see p. 261), and in order to avoid possible storage effect only freshly harvested tissue should be used. Uneven distribution of the vitamin in one individual fruit or vegetable (see p. 261) can usually be overcome by cutting a wedge from each of several units; and, to avoid enzymic oxidation of the vitamin, each wedge should be fed immediately after cutting. Cutting and mixing the full weight of sample before dosing should be avoided. When the effect of household cooking is being studied, the greatest care is needed to reproduce exactly the conditions of cooking from one day to another. If this precaution is not taken the composition of the feed may not be constant. However, with processed foods prepared on an industrial scale, it is usually relatively easy to ensure uniformity of both the raw material and the treatment to which it is subjected.

B. CHEMICAL METHODS

1. Preparation of Sample Extract

One of the most important features in the chemical estimation of ascorbic acid lies in the satisfactory preparation of the sample extract, and this is especially true of plant tissue. The main aim of the analyst is to effect complete extraction of the ascorbic acid in an unchanged form, i.e., without incurring loss by oxidation, at the same time reducing to a minimum the extraction of other substances which might interfere in the test reaction. In addition, care has to be taken that the material extracted is representative of the sample under test—an important consideration in view of the

[7] B. S. Gould and H. Schwachman, *J. Biol. Chem.* **151**, 439 (1943).

relatively small weight of material required and the wide variation in concentration of ascorbic acid within one plant organ, or between different individual fruits and vegetables.[6, 8, 8a, 8b] (See also p. 261.) Hand grinding with acid and sand is not only laborious and requires several extractions, but, since only a limited amount of material can be extracted by this procedure, it needs care to deal adequately with this difficulty of representative sampling.

The use of homogenizing or emulsifying apparatus, such as the Waring Blendor, has been valuable in helping to overcome this sampling difficulty as well as ensuring more complete extraction.[9-13] Emphasis has, however, been laid on the importance, during homogenizing, of avoiding the oxidation of ascorbic acid, which may be accelerated by the whipping of air into the acid-sample slurry (blending in an atmosphere of an inert gas has been suggested to avoid this[13-15]) or by the dissolution of copper from worn metal parts of the apparatus.[15] In addition to these precautions for avoiding oxidation of ascorbic acid by atmospheric oxygen during the extraction stage, the concentration of acid must be kept sufficiently high to inactivate oxidative enzymes set free during the process[14, 16-18] (usually 5 to 6 % metaphosphoric acid is preferred); similarly the tissue must not be minced, ground, or shredded except under the appropriate acid,[6, 17, 18] and it should be completely immersed in the extractant.[17, 19] Distilled water and other reagents should be free from copper.[17, 20, 21] To stabilize the vitamin, crystalline sodium chloride has also been suggested in place of sand when plant tissue is extracted by hand grinding.[21a] The ratio of extractant to

[8] M. Olliver, *Analyst* **63**, 2 (1938).

[8a] J. Whitacre, W. H. Brittingham, and L. C. Capp, *Southern Coöp. Ser. Bull.* **10**, 23 (1951).

[8b] W. J. Peterson, H. P. Tucker, J. T. Wakeley, R. E. Comstock, and F. D. Cochran, *Southern Coöp. Ser. Bull.* **10**, 13 (1951).

[9] W. B. Davis, *Ind. Eng. Chem. News Ed.* **17**, 752 (1939).

[10] S. A. Morell, *Ind. Eng. Chem. Anal. Ed.* **13**, 793 (1941).

[11] W. B. Davis, *Ind. Eng. Chem.* **34**, 217 (1942).

[12] L. Burkhart and R. A. Lineberry, *Food Research* **7**, 332 (1942).

[13] Association of Vitamin Chemists Inc., Methods of Vitamin Assay, 2nd ed., pp. 147–150. Interscience Publishers, New York, 1947.

[14] M. G. Vavich, R. M. Stern, and N. B. Guerrant, *Ind. Eng. Chem. Anal. Ed.* **17**, 531 (1945).

[15] M. P. Lamden, *Anal. Chem.* **22**, 1139 (1950).

[16] M. van Eekelen, *Nature* **136**, 144 (1935).

[17] R. R. Musulin and C. G. King, *J. Biol. Chem.* **116**, 409 (1936).

[18] N. C. Thornton, *Contribs. Boyce Thompson Inst.* **9**, 273 (1938).

[19] M. E. Reid, *Food Research* **7**, 288 (1942).

[20] G. L. Mack and Z. I. Kertesy, *Food Research* **1**, 377 (1936).

[21] R. W. Bell and T. J. Mucha, *J. Dairy Sci.* **32**, 827 (1949).

[21a] T. Marx, *Landwirtsch. Forsch.* **2**, 229 (1951).

sample is important, and between 7:1[22] and 4:1[23] has been recommended. Undoubtedly, however, the character of the sample must be considered. A dehydrated and starchy vegetable, for instance, requires a much higher ratio than a soft tissue sample.

Although trichloroacetic acid was initially recommended for the extraction of plant tissue,[24] this was subsequently replaced by metaphosphoric acid.[17, 20, 22, 25-28] This acid has the advantage of giving a clearer solution and also is a better stabilizer of ascorbic acid (especially in the presence of cuprous ions) than trichloroacetic acid. The use of other organic acids for this purpose has been investigated, and, of these, oxalic acid has been found as reliable as and even superior to metaphosphoric.[29-35] This acid, however, should be kept in the dark to avoid the formation of peroxides which may destroy ascorbic acid in the presence of catalysts.[36] If ferrous ions are present in the sample under test, the use of oxalic acid or metaphosphoric acid is likely to catalyze the reaction of these ions with 2,6-dichlorophenolindophenol unless suitable precautions are taken (see p. 249). Extraction with 10 % acetic acid containing 0.1 % oxalic acid, followed by reduction of pH to 0.4 before titration with the dye, has been suggested to avoid this difficulty,[37] and the use of sodium acetate-hydrochloric acid buffer of pH 0.65 has, for the same reason, been recommended when extracting stored canned foods.[38] Mineral acids have also been advised for samples where ferrous iron is likely to interfere, but some workers have found higher results when extracting with hydrochloric acid or sulfuric acid as compared with metaphosphoric acid. This finding, together with an apparent increase in ascorbic acid content observed by some workers when vegetables are cooked, has been attributed to the hydrolysis of protein bound or "combined" ascorbic acid.[39-46b] Other workers have been

[22] H. J. Loeffler and J. D. Ponting, *Ind. Eng. Chem. Anal. Ed.* **14**, 846 (1942).
[23] S. A. Goldblith and R. S. Harris, *Anal. Chem.* **20**, 649 (1948).
[24] L. J. Harris and S. N. Ray, *Biochem. J.* **27**, 303 (1933).
[25] A. Fujita and D. Iwatake, *Biochem. Z.* **277**, 293 (1935).
[26] C. M. Lyman, M. O. Schultze, and C. G. King, *J. Biol. Chem.* **118**, 757 (1937).
[27] O. A. Bessey, *J. Biol. Chem.* **126**, 771 (1938).
[28] W. L. Nelson and G. F. Somers, *Ind. Eng. Chem. Anal. Ed.* **17**, 754 (1945).
[29] J. H. Roe, *Science* **80**, 561 (1934); *J. Biol. Chem.* **116**, 609 (1936).
[30] B. Willberg, *Z. Untersuch. Lebensm.* **76**, 128 (1938).
[31] J. D. Ponting, *Ind. Eng. Chem. Anal. Ed.* **15**, 389 (1943).
[32] W. S. Gillam, *Ind. Eng. Chem. Anal. Ed.* **17**, 217 (1945).
[33] F. Wokes, *Analyst* **72**, 63 (1947).
[34] L. P. Guild, E. E. Lockhart, and R. S. Harris, *Science* **107**, 226 (1948).
[35] I. J. Babbar and B. Ahmad, *Ann. Biochem. and Exptl. Med. (India)* **9**, 103 (1949).
[36] F. E. Huelin, *Analyst* **75**, 391 (1950).
[37] F. E. Huelin and I. M. Stephens, *Australian J. Exptl. Biol. Med. Sci.* **25**, 17 (1947).
[38] F. Brown and W. B. Adam, *J. Sci. Food Agr.* **1**, 51 (1950).
[39] E. W. McHenry and M. L. Graham, *Nature* **135**, 871 (1935).

unable to confirm the presence of this complex[6, 16, 27, 47-50] and have attributed the apparent increase in both instances either to failure to inactivate the enzyme (thereby causing loss by oxidation) or to incomplete extraction of ascorbic acid or to lack of representative sampling.

It is now generally accepted that either metaphosphoric acid or oxalic acid is suitable for extraction in the assay of ascorbic acid, provided that precautions are taken along the lines already discussed and that, in the presence of interfering substances, the extract is suitably treated before titration (see pp. 249–250).

Having ground or blended the sample with acid, the slurry is either filtered or centrifuged, the latter procedure being necessary when quantities of colloidal substances are present, as, for example, with cooked potatoes. Complete extraction of ascorbic acid is essential. Pressing of the residue through muslin before filtration has therefore been advised,[6] and washing of the residues is similarly to be emphasized.[27] The ascorbic acid present in the extract is then determined by one or other methods to be described.

For animal tissues in general, hand grinding with sand and concentrated metaphosphoric acid has been found satisfactory. Special treatment, however, is necessary with some samples, and with blood, for instance, lithium oxalate is added to prevent clotting. The plasma obtained from the oxalated blood is at once deproteinized with metaphosphoric acid and assayed.[13] In collecting urine for estimation of vitamin C, a small amount of glacial acetic acid or metaphosphoric acid is added to retard loss by oxidation, and the subsequent titration is then generally carried out without further treatment, except for such dilution or addition of acid as may be necessary.

2. Estimation of Ascorbic Acid in Sample Extract

Numerous chemical methods have been suggested for the determination of ascorbic acid, the majority being based upon colorimetric changes

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[40] E. W. McHenry and M. L. Graham, *Biochem. J.* **29**, 2013 (1935).
[41] B. C. Guha and J. C. Pal, *Nature* **137**, 946 (1936).
[42] B. C. Guha and P. N. Sen-Gupta, *Nature* **141**, 974 (1938).
[43] E. J. Reedman and E. W. McHenry, *Biochem. J.* **32**, 85 (1938).
[44] H. Scarborough and C. P. Stewart, *Nature* **142**, 40 (1938).
[45] F. De Eds, *Food Research* **8**, 275 (1943).
[46] W. K. Schwarze and E. Gunther, *Pharmazie* **4**, 161 (1947).
[46a] W. N. Sumerwell and R. R. Sealock, *J. Biol. Chem.* **196**, 753 (1952).
[46b] Z. Prochazka and S. Koristek, *Collection Czechoslov. Chem. Communs.* **16**, 65 (1951).
[47] G. L. Mack, *Nature* **138**, 505 (1936).
[48] G. L. Mack and D. K. Tressler, *J. Biol. Chem.* **118**, 735 (1937).
[49] M. Hochberg, D. Melnick, and B. L. Oser, *Ind. Eng. Chem. Anal. Ed.* **15**, 182 (1943).
[50] F. Sargent and R. Golden, *J. Biol. Chem.* **188**, 773 (1951).

when the sample solution, with or without preliminary treatment, is titrated against an indicator. Among the substances recommended for this purpose are 2,6-dichlorophenolindophenol (2,6-dichlorobenzenonindophenol), iodine, 2,4-dinitrophenylhydrazine, methylene blue,[51-54] phosphotungstic acid,[55] phosphomolybdic acid,[55a] *p*-sulfophenylhydrazine,[55b] uranium nitrate,[55c] silicomolybdic acid,[56] potassium ferricyanide,[57] diazotized sulfanilamide,[58] ferridipyridyl,[59] and thionine.[60]

Many of these methods have received but little application, mainly because of their lack of specificity, and attention has been essentially concentrated on the reagents 2,6-dichlorophenolindophenol, 2,4-dinitrophenylhydrazine, and, to a lesser extent, iodine. The use of 2,4-dinitrophenylhydrazine is of special interest in connection with the estimation of dehydroascorbic acid and will therefore be dealt with under the appropriate section (see p. 251). The reaction of iodine with ascorbic acid has been made the basis of several methods, and apart from direct titration with iodine[61] the use of iodate[62] and similar compounds has been suggested. These methods are simple and easy to use, but iodine is so relatively strong an oxidizing agent that it will also react with substances, other than ascorbic acid, e.g., glutathione, which may be present in the sample extract; therefore it is mainly of value in testing pure ascorbic acid or pharmaceutical preparations free from interfering substances.

In 1927 Tillmans published the results of his investigations into the estimation of oxidation-reduction potentials and their application to food chemistry.[63] An especially interesting observation was the strong reducing power shown by fresh lemon and other fruit juices against 2,6-dichlorophenolindophenol, and Tillmans used this as a means of distinguishing

[51] E. Martini and A. Bonsignore, *Boll. soc. ital. biol. sper.* **9**, 388 (1934).
[52] H. Lund and H. Lieck, *Nature* **137**, 784 (1936).
[53] T. Guthe and K. K. Nygaard, *Chemistry & Industry* **57**, 1195 (1938).
[54] A. M. Butler, M. Cushman, and E. A. MacLachlan, *J. Biol. Chem.* **150**, 453 (1943).
[55] A. Fujita and T. Ebihara, *Biochem. Z.* **290**, 182 (1937).
[55a] S. Camozzo, *Ann. chim. (Rome)* **41**, 188 (1951).
[55b] J. Baraud, *Bull. soc. chim. (Paris)* **18**, 837 (1951).
[55c] M. Z. Barakat, N. Badran, and S. K. Shehab, *J. Pharm. and Pharmacol.* **4**, 46 (1952).
[56] M. L. Isaacs, *Ind. Eng. Chem. Anal. Ed.* **14**, 948 (1942).
[57] H. Tauber and J. S. Kleiner, *J. Biol. Chem.* **108**, 563 (1935).
[58] J. V. Scudi and H. D. Ratish, *Ind. Eng. Chem. Anal. Ed.* **10**, 420 (1938).
[59] R. A. Koenig, T. L. Schiefelbusch, and C. R. Johnson, *Ind. Eng. Chem. Anal. Ed.* **15**, 181 (1943).
[60] J. K. Wanka and V. Herout, *Chem. Listy.* **43**, 58 (1949).
[61] J. W. Stevens, *Ind. Eng. Chem. Anal. Ed.* **10**, 269 (1938).
[62] R. Ballentine, *Ind. Eng. Chem. Anal. Ed.* **13**, 89 (1941).
[63] J. Tillmans, *Z. Untersuch. Lebensm.* **54**, 33 (1927).

natural from imitation fruit juices. In a later communication[64] he reported biological tests which showed that this reduction of the dye to the leuco form was a measure of the antiscorbutic activity of the lemon juice. Subsequent observations by Tillmans and his associates[65-67] confirmed this relationship, which was used by L. J. Harris and his coworkers for the development of a specific quantitative test for the estimation of ascorbic acid.[24, 68] Their more important modifications included the use of a preliminary extraction process with trichloroacetic acid (later replaced by metaphosphoric acid), titration in relatively strong acid solution, and rapid completion of titration (1 to 2 minutes), whereby the interfering action of the majority of reducing substances other than ascorbic acid is inhibited. Bessey and King[69] about this time put forward similar suggestions, the main difference in procedure from the Harris method being that they preferred to titrate the dye against a fixed volume of sample extract and not vice versa. This is possibly a point of personal preference, depending upon whether discharge or appearance of the pink color is found to be the more readily detected.

It is generally accepted that the pH at which the titration is carried out should lie between 1.0 and 3.5, although Bessey quotes a range of 2.0 to 3.0 as a necessary condition for specificity.[70] At pH 1.0 some slight fading of the dye occurs, but it has been shown that interference by sulfhydryl groups is eliminated at this point.[71] Because of the importance of the time factor, a microtitration is usually preferred; 0.05 ml. of dye, equivalent to approximately 0.02 mg. of ascorbic acid, is titrated with the sample extract of such concentration that approximately 1 to 2 ml. is taken. In order to maintain a relatively constant titer, the use of graded concentrations of both sample extract and dye have been recommended for different ascorbic acid levels.[6] The dye solution is usually prepared in a concentration of 0.025% to 0.100%,[6, 13] and the addition of small quantities of sodium bicarbonate[13] or buffer at pH 6.8[69] has been suggested by some workers. Storage in the dark and at a low temperature is advised, and renewal at weekly intervals is usually recommended.[13, 69] Daily standardization is essential. This may be carried out by titration against pure ascorbic acid in metaphosphoric acid[13] or against ferrous salts[72] or by the reaction of the

[64] J. Tillmans, *Z. Untersuch. Lebensm.* **60**, 34 (1930).
[65] J. Tillmans, P. Hirsch, and W. Hirsch, *Z. Untersuch. Lebensm.* **63**, 1 (1932).
[66] J. Tillmans, P. Hirsch, and J. Jackisch, *Z. Untersuch. Lebensm.* **63**, 241 (1932).
[67] J. Tillmans, P. Hirsch, and J. Jackisch, *Z. Untersuch. Lebensm.* **63**, 276 (1932).
[68] T. W. Birch, L. J. Harris, and S. N. Ray, *Biochem. J.* **27**, 590 (1933).
[69] O. A. Bessey and C. G. King, *J. Biol. Chem.* **103**, 687 (1933).
[70] O. A. Bessey, *J. Am. Med. Assoc.* **111**, 1290 (1938).
[71] C. G. King, *Ind. Eng. Chem. Anal. Ed.* **13**, 225 (1941).
[72] A. J. Lorenz and L. J. Arnold, *Ind. Eng. Chem. Anal. Ed.* **10**, 687 (1938).

dye with potassium iodide to produce free iodine, which is then titrated with sodium thiosulfate solution.[73-75]

a. Comparison between Results from Indophenol Titration and Biological Tests

The adoption of 2,6-dichlorophenolindophenol as the basic indicator for the chemical assay of ascorbic acid has undoubtedly been justified in the case of fresh fruit and vegetables and the majority of products prepared from them, and the accuracy has been confirmed by comparison of results against biological test.[6, 24, 65-67, 69, 76-83, 83a]

However, it was recognized during the development of this chemical method that, in special cases, small amounts of the biologically active reversibly oxidized form of ascorbic acid, dehydroascorbic acid, might be present and would not be estimated by the indophenol method as normally applied. In addition, it was appreciated that, in some exceptional instances, the samples might also contain substances, not ascorbic acid, which might reduce the dye. Although in general, therefore, the 2,6-dichlorophenolindophenol method can be used with confidence for the estimation of ascorbic acid, suitable modifications have to be made in these exceptional cases where either dehydroascorbic acid or interfering substances are present. These possible complications have been studied in some considerable detail by many workers, and various alternative modifications have been proposed.

b. Dehydroascorbic Acid

In canned and fresh fruits and vegetables, dehydroascorbic acid is usually present only in very small amounts or else in an unstable condition.[6, 83a-87] Under some conditions, however, it would appear that significant quantities of dehydroascorbic acid may be produced.[83a, 86, 88]

[73] R. E. Buck and W. S. Ritchie, *Ind. Eng. Chem. Anal. Ed.* **10,** 26 (1938).
[74] H. Dick, *Ind. Eng. Chem. Anal. Ed.* **11,** 293 (1941).
[75] M. H. Menaker and N. B. Guerrant, *Ind. Eng. Chem. Anal. Ed.* **10,** 25 (1938).
[76] L. J. Harris and S. N. Ray, *Biochem. J.* **27,** 580 (1933).
[77] H. Lund, B. Spur, and L. S. Fridericia, *Biochem J.* **28,** 1825 (1934).
[78] L. F. Levy and F. W. Fox, *Biochem. J.* **29,** 884 (1935).
[79] A. E. Kellie and S. S. Zilva, *Biochem. J.* **30,** 1216 (1936).
[80] M. Olliver, *J. Soc. Chem. Ind.* **55,** 153T (1936).
[81] E. Mathiesen and V. Aschehoug, *Arch. Math. Naturvidenskab* **41,** No. 8 (1937).
[82] L. J. Harris and L. W. Mapson, *Brit. J. Nutrition* **1,** 7 (1947).
[83] L. J. Harris, L. W. Mapson, K. E. Kodicek, T. Moore, and V. H. Booth, *Proc. 11th Intern. Congr. Pure and Appl. Chem., London* (1947).
[83a] E. M. Hewston, M. Fisher and E. Orent-Keiles, *U. S. Dept. Agr., Tech. Bull.* **1023,** 30 (1951).
[84] W. Stone, *Biochem. J.* **31,** 508 (1937).

(1) *Hydrogen Sulfide.* Tillmans[89, 90] and other workers[27, 39, 91, 92] showed that, whereas 2,6-dichlorophenolindophenol did not measure this oxidized form, sample extracts could be reduced with hydrogen sulfide, which was removed before titration of the total ascorbic acid. Several investigators [48, 71, 86, 93] have shown, however, that this method cannot be applied to some types of sample, owing to the possibility of production by the hydrogen sulfide of substances, other than ascorbic acid, which may react with the dye.

(2) *2,4-Dinitrophenylhydrazine.* Subsequently, Roe and Kuether[94] developed a method which, they claimed, measured dehydroascorbic acid as well as reduced ascorbic acid in blood and urine. Oxidation is effected by Norit, and then the oxidation products are condensed with 2,4-dinitrophenylhydrazine. The osazone so produced is soluble in sulfuric acid, giving a red color which can be measured photometrically, the intensity being proportional to the quantity of ascorbic acid originally present, together with any dehydroascorbic acid. Acetic acid was subsequently suggested as a substitute for sulfuric acid,[95] but it is less effective in inhibiting interfering substances.[96] Roe and Oesterling adapted the Roe and Kuether method for the determination of dehydroascorbic acid in the presence of ascorbic acid in plant tissue.[85] Oxidation with Norit was omitted, and thiourea was added to stabilize the reduced form. Substances other than dehydroascorbic acid, such as reductones and the biologically inactive 2,3-diketo-L-gulonic acid,[97] also react with 2,4-dinitrophenylhydrazine, and the method has been found not specific for aerated orange juice[98] and stored solutions of dehydroascorbic acid and reduced ascorbic acid.[34] Guild *et al.*[34] have made the interesting suggestion that the Roe and Kuether method may successfully be applied to oxalic acid slurries of garden fresh fruits or vegetables which have had to be stored or transported before assay, since decomposition products of the original dehydroascorbic acid

[85] J. H. Roe and M. J. Oesterling, *J. Biol. Chem.* **152**, 511 (1944).
[86] M. C. Miller, *Food Research* **12**, 343 (1947).
[87] F. C. Lamb, L. D. Lewis, and D. G. White, *Food Technol.* **5**, 269 (1951).
[88] T. J. McMillan and E. N. Todhunter, *Science* **103**, 196 (1946).
[89] J. Tillmans, P. Hirsch, and F. Siebert, *Z. Untersuch. Lebensm.* **63**, 21 (1932).
[90] J. Tillmans, P. Hirsch, and H. Dick, *Z. Untersuch. Lebensm.* **63**, 267 (1932).
[91] A. Emmerie, *Biochem. J.* **28**, 268 (1934).
[92] M. Van Eekelen and A. Emmerie, *Biochem. J.* **30**, 25 (1936).
[93] W. W. Woessner, C. A. Elvehjem, and H. A. Schuette, *Nutrition* **20**, 327 (1940).
[94] J. H. Roe and C. A. Kuether, *J. Biol. Chem.* **147**, 399 (1943).
[95] R. A. Bolomey and A. R. Kemmerer, *J. Biol. Chem.* **165**, 377 (1946).
[96] M. B. Mills and J. H. Roe, *J. Biol. Chem.* **170**, 159 (1947).
[97] J. R. Penney and S. S. Zilva, *Biochem. J.* **37**, 403 (1943).
[98] M. Pijoan and H. J. Gerjovitch, *Science* **103**, 202 (1946).

will also be determined. Experimental data to confirm the validity of this proposal have been put forward by Goldblith and R. S. Harris.[23]

Roe *et al.*[99] have developed a method for the simultaneous determination of ascorbic acid, dehydroascorbic acid, and diketogulonic acid in the one sample extract. Stannous chloride is used so that the ascorbic acid in a metaphosphoric acid extract is stabilized and does not react with 2,4-dinitrophenylhydrazine, which is then used to determine the dehydroascorbic acid and diketogulonic acid content. The dehydroascorbic acid is then reduced by hydrogen sulfide and the diketogulonic acid content, which is not affected, is determined by the dinitrophenylhydrazine. After removal of hydrogen sulfide from the filtrate, bromine is used to yield a solution containing all the oxidized forms of the ascorbic acid. The value of this method when testing aged and some processed foodstuffs has been emphasized in a subsequent publication.[100] The advantage of substituting thiourea for stannous chloride in the stabilization of ascorbic acid has recently been claimed.[87] Lowry *et al.* adapted the dinitrophenylhydrazine test for the determination of ascorbic acid in amounts as small as 0.01 ml. of serum.[101] This micro method has been found reliable for urine and blood and has the advantage of allowing ascorbic acid determinations in whole blood[98, 102, 103] Attention has, however, been drawn to the necessity of adopting suitable precautions against possible extraction of interfering substances from rubber stoppers.[104]

(3) *Bacteria.* A proposed improvement over the hydrogen sulfide method for dehydroascorbic acid, with regard to both specificity and time involved, was suggested by Gunsalus and Hand, who used *Escherichia coli* to reduce dehydroascorbic acid in fruit juices, milk, and urine, although they found this inapplicable to some vegetable extracts.[105] Stewart and Sharp, however, satisfactorily used a strain of *Staphylococcus albus* for a large number of vegetable juices as well as for milk, fruit juices, urine, and blood plasma.[106] They also reported that *E. coli* could be used for the quantitative determination of dehydroascorbic acid in vegetable extracts, if the pH of titration were kept above 4.5 after inactivation of the bacteria. A pH of 3.5 or

[99] J. H. Roe, M. B. Mills, M. J. Oesterling, and C. M. Damron, *J. Biol. Chem.* **174**, 201 (1948).

[100] M. B. Mills, C. M. Damron, and J. H. Roe, *Anal. Chem.* **21**, 707 (1949).

[101] O. H. Lowry, J. A. Lopez, and O. A. Bessey, *J. Biol. Chem.* **160**, 609 (1945).

[102] B. B. Lloyd, H. M. Sinclair, and G. R. Webster, *Proc. Biochem. J.* **49**, XVII (1945)

[103] W. Daubenmerkl, *Acta Pharmacol. Toxicol.* **5**, 270 (1949).

[104] R. L. Goodland, R. S. Sealock, N. S. Scrimshaw, and L. C. Clark, *Science* **109**, 494 (1949).

[105] I. C. Gunsalus and D. B. Hand, *J. Biol. Chem.* **141**, 853 (1941).

[106] A. P. Stewart and P. F. Sharp, *Ind. Eng. Chem. Anal. Ed.* **17**, 373 (1945).

less could be used satisfactorily only in the presence of sodium cyanide. Mapson and Ingram, applying this modified *E. coli* method, could not obtain complete recovery of dehydroascorbic acid, owing to a small concurrent conversion to diketogulonic acid.[107] They showed, however, that this can be allowed for by calculation and is minimized by the use of sodium azide in place of the cyanide. These workers traced the failure of the *E. coli* method in vegetable juices to the formation of nitrous acid from nitrates by the nitratase enzyme system of the bacteria and were able to overcome this interference by using amidosulfonic acid. D-Isoascorbic acid, reductic, and hydroxytetronic acids, if present, would cause interference in this method.

c. Reducing Substances Not Ascorbic Acid

The 2,6-dichlorophenolindophenol method, if carried out with the precautions already described, is usually not subject to interference by reducing substances other than ascorbic acid. Glutathione, for instance, and some phenolic substances are prevented from interfering by the fact that the titration is carried out in strongly acid solution.[24, 68] Sulfur dioxide, ferrous salts, and tannins are, however, among the reducing substances for which suitable modifications may have to be made in the determination of ascorbic acid by the indophenol method. Two approaches to this problem have been suggested. One is to titrate the sample extract before and after the vitamin has been destroyed by ascorbic acid oxidase.[108, 109] This principle has received relatively little application, mainly because the enzyme is not fully specific for ascorbic acid, and attention has been concentrated on the alternative procedure, which is adjustment of conditions of assay so that substances which may normally reduce the dye are inhibited or allowed for by difference.

(1) *Tannins, Cysteine, Thiosulfate, and Sulfhydryl Compounds.* Van Eekelen and Emmerie removed tannins, cysteine, thiosulfate, and sulfhydryl compounds with mercuric acetate, followed by precipitation of the excess mercury salt with hydrogen sulfide which, it was claimed, also allowed the estimation of dehydroascorbic acid.[92] This procedure was also found to have the added advantage of removing color likely to interfere in the determination of the end point when titrating plant extracts.

In the urine of subjects on low intakes of vitamin C, a considerable proportion of the indophenol titer may be due to thiosulfate rather than to ascorbic acid itself. With higher intakes of the vitamin, however, the indophenol-reducing substance is mostly ascorbic acid. This consideration does

[107] L. W. Mapson and M. Ingram, *Biochem. J.* **48**, 551 (1951).
[108] M. Srinivasan, *Biochem. J.* **31**, 1524 (1937).
[109] G. T. Meiklejohn and C. P. Stewart, *Biochem. J.* **35**, 761 (1941).

not affect the validity of the procedure of determining human nutritional status in vitamin C by counting the number of days of test dosing needed to bring about a state of saturation, for the amount of ascorbic acid excreted on attaining saturation is out of all proportion higher than the non-specific "blank correction" before saturation.[109a]

(2) *Tin, Iron, and Sulfur Dioxide.* Tin, although reacting with the dye, is normally present in insufficient quantities to cause interference, but sulfur dioxide and ferrous iron, both of which reduce 2,6-dichlorophenol-indophenol, are relatively more common causes of difficulty, the former by reason of its use as a preservative of foodstuffs and the latter because of the possibility of its presence in stored canned fruits and vegetables and in pharmaceutical products. To eliminate the effect of sulfur dioxide, removal of the gas by means of a current of nitrogen and exhaustion *in vacuo* has been used.[110] Hydrogen peroxide has been suggested as a means of oxidation of the sulfur dioxide before titration, but low values for ascorbic acid have been found when this method of assay was used in the presence of copper and ferrous ions.[111] Mapson proposed the use of acetone for inhibiting the effect of sulfur dioxide,[110] and the reliability of this procedure was confirmed by Loeffler and Ponting.[22] Huelin found that the titration lacked sharpness at the pH specified (1.5) and preferred 2.5.[112]

The interference of ferrous ions may be prevented by the use of suitable extractants for the sample (see p. 249), by passing an oxalic acid extract through a column of an ion exchange resin,[112a] or by treatment with hydrogen peroxide or formaldehyde before titration of the metaphosphoric acid or oxalic acid extract. Chapman *et al.*[113] have found the method of Brown and Adam[38] to be the most suitable of several procedures tested for the routine estimation of pharmaceutical products containing ferrous iron, but when copper is also present they recommend the method of Roe *et al.*[99]

(3) *Reductones and Allied Substances.* Cocoa,[114] kaffir beer,[115] yeast,[93] malt extracts,[116] molasses,[114] scorched and some stored dehydrated vegetables,[117] honey,[86] and walnuts[118] have all been found to reduce the dye, under normal conditions of test, to a degree which is not commensurate

[109a] L. J. Harris, *Lancet* **I**, 642, 644 (1942); **I**, 515 (1943).
[110] L. W. Mapson, *Biochem. J.* **36**, 196 (1942).
[111] L. F. Levy, *Nature* **152**, 599 (1943); *Biochem. J.* **37**, 713 (1943).
[112] F. E. Huelin, *Australian Chem. Inst. J. & Proc.* **14**, 498 (1947).
[112a] T. L. Parkinson, *J. Sci. Food Agr.* **3**, 555 (1952).
[113] D. G. Chapman, O. Rochon, and J. A. Campbell, *Anal. Chem.* **23**, 1113 (1951).
[114] F. Wokes, J. G. Organ, J. Duncan, and F. C. Jacoby, *Nature* **152**, 14 (1943).
[115] F. W. Fox and W. Stone, *Nature* **140**, 234 (1937).
[116] L. J. Harris, *Nature* **132**, 27 (1933).
[117] L. W. Mapson, *J. Soc. Chem. Ind.* (*London*) **62**, 223 (1943).
[118] R. Melville, F. Wokes, and J. G. Organ, *Nature* **152**, 447 (1943).

with their true ascorbic acid content and cannot be accounted for by the interfering substances already mentioned. The interference in most of these instances can be attributed either to reductones (which can be produced by heating sugar solutions with alkali in the absence of air) or possibly to substances resembling reductic acid (produced by heating galacturonic acid, pectin, or xylose with mineral acid).

To differentiate ascorbic acid from these interfering substances, Lugg made use of the fact that the vitamin reacts rapidly with formaldehyde at pH 3.5 but slowly at pH 1.5.[119] Other indophenol-reducing substances, such as sulfite and cysteine, condense rapidly with the formaldehyde at both pH 3.5 and pH 1.5, whereas reductones, thiourea, and ferrous salts were found not readily to do so at either pH. Titration before and after condensation with formaldehyde at pH 3.5 and pH 1.5 was therefore recommended.

Mapson, however, found that reductones and reductone-like substances in caramelized, fermented foods and stored dehydrated vegetables did, in fact, combine fairly rapidly at pH 3.5 but only slowly at pH 2.0 in the presence of 8% formaldehyde, and he was therefore able to suggest a suitable modification to Lugg's method.[117, 120] By a slight adjustment, dihydroxymaleic acid and the reducing substances present in malt and beet molasses could also be differentiated from ascorbic acid. Snow and Zilva,[121] on the other hand, concluded that, with alkali-treated glucose solution, no greater accuracy could be obtained by titrating at pH 2.0 instead of at 3.5, and they suggested a procedure which more closely approached Lugg's original method. Further modifications were also put forward by Wokes *et al.*[122] No simple quantitative method has been described for the determination of, and hence allowance for, hydroxytetronic acid or isoascorbic acid in the presence of ascorbic acid, but there is no evidence that either occurs in natural products. It seems certain that further chromatographic studies will furnish alternative methods to differentiate these substances so closely related to ascorbic acid.[123]

L. J. Harris and colleagues have devised a method which makes use of a continuous flow apparatus.[82, 83, 123a] It was determined that, under suitably controlled conditions, the rate of reaction of interfering substances with the

[119] J. W. H. Lugg, *Nature* **150**, 577 (1942); *Australian J. Exptl. Biol. Med. Sci.* **20**, 273 (1942).

[120] L. W. Mapson, *Nature* **152**, 13 (1943).

[121] G. A. Snow and S. S. Zilva, *Biochem. J.* **38**, 458 (1944).

[122] F. Wokes, J. G. Organ, and F. C. Jacoby, *J. Soc. Chem. Ind.* (*London*) **62**, 232 (1943).

[123] L. W. Mapson and S. M. Partridge, *Nature* **164**, 479 (1949).

[123a] M. N. Bland, B. J. Constable, L. J. Harris, and R. E. Hughes, *Proc. 12th Intern. Congr. Pure and Appl. Chem., New York* (1951).

dye is different from and hence can be distinguished from ascorbic acid. Good agreement was found between results obtained by the "continuous flow" and formaldehyde methods when fresh, processed, and caramelized foods were tested. Harris and Mapson[82] made an important contribution to this field of study in obtaining confirmation of the validity of both these methods by biological assays.

(4) *In Pigmented Solution.* The presence of anthocyanins or other natural coloring matters in tissue extracts may obscure the colorimetric end point in the indophenol titration method. To overcome this difficulty and to avoid the personal element in the visual titration of ascorbic acid, electrometric titration has been used by Kirk and Tressler, working with vegetable and fruit extracts.[124] A continuous drift in potential during titration with the dye made the potentiometric method extremely difficult to operate, but L. J. Harris *et al.* obtained considerable improvement by using a platinum and mercury electrode.[125] This was, however, unsuitable in the presence of high concentrations of reducing substances other than ascorbic acid and, in such instances, a bright platinum electrode was recommended.

Various photoelectric colorimeters have been applied with considerable success to the estimation of ascorbic acid and have fulfilled the dual purpose of correcting for the natural color and for spontaneous slow fading of the dye in acid solution. Mindlin and Butler,[126] working with blood serum, and Bessey,[27] with plant tissue, found that addition of excess 2,6-dichlorophenolindophenol with instantaneous measurement of the residual color in a photocolorimeter avoided interference from the slower reducing nonspecific substances.

Their work, based on the discussions of Rosen and Evelyn,[127] confirmed the observations of Meunier[128] which appeared about this time. Bessey also showed that, with a photoelectric colorimeter, satisfactory results were obtained, even in the presence of anthocyanins or in turbid solution, when buffers at pH 3.5 to 3.7 were used. This photoelectric method of assay, with various modifications, has been applied to several colorimeters and to a wide range of materials.[10, 22, 49, 129-132]

Oxidized 2,6-dichlorophenolindophenol can be quantitatively extracted

[124] M. M. Kirk and D. K. Tressler, *Ind. Eng. Chem. Anal. Ed.* **11,** 322 (1939).
[125] L. J. Harris, L. W. Mapson, and Y. L. Wang, *Biochem. J.* **36,** 183 (1942).
[126] R. L. Mindlin and A. M. Butler, *J. Biol. Chem.* **122,** 673 (1937–1938).
[127] C. Rosen and K. A. Evelyn, *Proc. Roy. Soc. Can.* **31,** Appendix B, cliii (1937).
[128] P. Meunier, *Bull. soc. chim. biol.* **19,** 877 (1937).
[129] K. A. Evelyn, H. T. Malloy, and C. Rosen, *J. Biol. Chem.* **126,** 645 (1938).
[130] M. A. Elliott, A. L. Sklar, and S. F. Acree, *J. Research Natl. Bur. Standards* **26,** 117 (1941).
[131] C. Carruthers, *Ind. Eng. Chem. Anal. Ed.* **14,** 826 (1942).
[132] A. McN. Taylor, *Biochem. J.* **37,** 54 (1943).

from acid solution by xylene and other solvents in which water-soluble pigments are insoluble, and use can be made of this finding for visual and photometric assay of ascorbic acid in colored extracts of plant tissue and blood and urine.[6, 28, 40, 133-135] Care should be exercised in the choice of xylene, since some batches have been found to introduce errors through the presence of oxidizing substances.[28] Rubin *et al.*[136] described a method which also determined dehydroascorbic acid, and Robinson and Stotz[137] suggested the use of formaldehyde and hydrogen peroxide to allow for the presence of reductones and other interfering substances.

A further complication has recently been found with some colored extracts, since it has been shown that the biologically inactive anthocyanin betanin in red beet also reacts with the dye in the indophenol-xylene extraction method, even in the presence of formaldehyde.[138] It has also been reported that, in some anthocyanin-containing berries, a high percentage of the total apparent ascorbic acid is not fixed by formaldehyde.[139] This finding, which is not in accordance with the work of, for instance, Miller,[86] is somewhat surprising in view of the good agreement shown between the results from biological and chemical assay on fresh fruits and vegetables (see p. 250), including some of the fresh berries cited and stored products made from the fruit.[6, 80] Further experimental data from biological tests would undoubtedly be valuable for assessing the reliability of these various methods which have been proposed for the estimation of ascorbic acid in the presence of interfering substances.

C. PHYSICAL METHODS

The relative simplicity of the chemical methods for estimation of ascorbic acid has diverted interest from the possible application of physical techniques in this field. However, Kodicek and Wenig reported a successful modification of the polarographic procedure for the estimation of the vitamin by making use of oxidation at a dropping mercury anode, instead of the more usual reduction at a dropping mercury cathode.[140] Investigations by other workers into the use of the polarograph in the assay of ascorbic acid in natural products were encouraging.[141-143] Gillam subse-

[133] F. Bukatsch, Hoppe-Seyler's *Z. physiol. Chem.* **262**, 20 (1939).
[134] E. Stotz, *J. Lab. Clin. Med.* **26**, 1542 (1940–1941).
[135] L. P. Pepkowitz, *J. Biol. Chem.* **151**, 405 (1943).
[136] S. H. Rubin, F. W. Jahns, and J. C. Bauernfeind, *Fruit Products J.* **24**, 327 (1945).
[137] W. B. Robinson and E. Stotz, *J. Biol. Chem.* **160**, 217 (1945).
[138] G. F. Somers, W. C. Kelly, E. J. Thacker, and A. M. Redder, *Science* **110**, 17 (1949).
[139] G. F. Somers, W. C. Kelly, E. J. Thacker, and A. M. Redder, *Food Research* **16**, 62 (1951).
[140] E. Kodicek and K. Wenig, *Nature* **142**, 35 (1938).
[141] K. Schwarz, *Z. anal. Chem.* **115**, 161 (1938–1939).

quently described a method whereby the apparatus was used with a number of fruit and vegetable extracts.[32] It was found that good agreement was obtained between results from the polarograph on the one hand and the visual titration and photometric methods on the other, although with some materials, e.g., dehydrated potatoes, the colloidal content of the extract interfered. It was recognized that the method failed in the presence of a high content of interfering substances, but since their presence can be detected by inspection of the anodic wave, it was claimed that the method would, in this respect, appear to have advantages over the "straight" titration methods. Various modifications along the lines adopted for the keto steroids have been suggested for improving the accuracy of the method of assay.[144] At present, however, the use of the polarograph for the estimation of ascorbic acid is limited, since, for instance, Mackenzie has noted that changes in viscosity produced by substances in true solution significantly affect the diffusion currents.[145] Polarography seems to have no advantage over the simpler method of chemical titration, not even as regards specificity.

Spectrophotometric methods have also been suggested, although it has been shown that, beyond specified limits, the intensity of absorption is not directly proportional to the concentration of ascorbic acid.[146] Another disadvantage is that the well-defined absorption band of ascorbic acid is subject to interference from many other substances in this region. Johnson attempted to overcome this by determining the characteristic absorption spectra before and after destruction of the ascorbic acid with cuprous ions.[147] The same principle has been adopted by other workers, but using means other than cuprous ions for destruction of the vitamin, e.g., irradiation[148, 149] and ascorbic acid oxidase.[150] The effect of pH in altering the position of the absorption band of ascorbic acid, without necessarily moving those of the interfering substances, has been used as another means of spectrophotometric assay, measurement being made at two or more levels of pH.[151, 152]

A spectrophotometric method which is based on the color reaction be-

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[142] T. Østerud, *Tek. Ukeblad* **86**, 216 (1939).
[143] D. Cozzi, *Ann. chim. appl.* **29**, 434 (1939).
[144] J. E. Page and J. G. Waller, *Analyst* **71**, 65 (1946).
[145] H. A. McKenzie, *J. Council Sci. Ind. Research* **21**, 210 (1948).
[146] R. W. Herbert, E. L. Hirst, E. G. V. Percival, R. J. W. Reynolds, and F. Smith. *J. Chem. Soc.* **1933**, 1270.
[147] S. W. Johnson, *Biochem. J.* **30**, 1430 (1936).
[148] A. Chevallier and Y. Choron, *Bull. soc. chim. biol.* **19**, 511 (1937).
[149] A. Chevallier and Y. Choron, *Compt. rend. soc. biol.* **124**, 453 (1937).
[150] A. Fujita and T. Sakamoto, *Biochem. Z.* **297**, 10 (1938).
[151] M. Vacher and D. Faucquembergue, *Bull. soc. chim. biol.* **31**, 1419 (1949).
[152] C. Daglish, *Biochem. J.* **49**, 635 (1951).

tween ascorbic acid and either perinaphthindanetrione hydrate or the derivative 2-nitroperinaphthindanetrione hydrate has recently been proposed.[153] Reductones, reductic acid, and cysteine may cause interference, but it is claimed that, for the estimation of ascorbic acid in pure solutions, blood plasma, and urine, this method is as accurate as, but simpler and quicker in operation than, the Roe and Kuether method.

Further developments of physical methods for the estimation of ascorbic acid are awaited with interest. It is doubtful, however, whether any of those already described will have wide application, except possibly for pharmaceutical preparations, unless they are modified to give greater specificity compared with the chemical methods of ascorbic acid assay.

VI. Standardization of Activity

MAMIE OLLIVER

In 1931 the Permanent Commission on Biological Standardization of the League of Nations adopted 0.1 ml. of freshly expressed lemon juice, *Citrus limonum*, as the unit of vitamin C activity. The possible variation in ascorbic acid content of this juice was subsequently emphasized however,[1] and in 1934 the Commission recommended the adoption of L-ascorbic acid as a standard of reference. However, in order to maintain the biological value of the unit, the Commission approved 0.05 mg. of L-ascorbic acid as the international unit (I.U.) of vitamin C activity, since this is the approximate average content of 1 ml. of lemon juice. Hence, by definition 1 g. of L-ascorbic acid is equivalent to 20,000 I.U.

The criteria of purity laid down for this international standard by the International Conference in 1934 and the findings of Haworth on a sample prepared by Szent-Györgyi are quoted by Coward:[2]

	I.C.	Haworth
Melting point	192°	193°
Specific rotation in water $[\alpha]_D^{20}$	+22.4°	+22.6°
(Concentration, g./ml.	2.2	2.34)
Amount in milliliters of 0.01 *N* aqueous iodine required by 10 mg., the titration being carried out with starch as indicator	11.4	11.4

[153] M. S. El Ridi, R. Moubasher, and Z. F. Hassan, *Biochem. J.* **49**, 246 (1951).

[1] A. L. Bacharach, P. M. Cook, and E. L. Smith, *Biochem. J.* **28**, 1038 (1934).

[2] K. H. Coward, Biological Standardization of the Vitamins, 2nd ed., p. 87. Baillière, Tindall and Cox, London, 1947.

Absorption spectrum in slightly acid aqueous solution characterized by a single intense band with head at wavelength	245 mμ	245 mμ
Molecular extinction coefficient	10,000	10,000
Ultimate analysis, C	40.9%[a]	40.95%
Ultimate analysis, H	4.6%[a]	4.6%

[a] Theoretical.

Further detailed data (some being more recent) are as follows:[3]

Empirical formula	$C_6H_8O_6$
Molecular weight	176
Melting point	192°
Acidic dissociation pK_1	4.2
Acidic dissociation pK_2	11.6
Solubility	Soluble in water, methyl alcohol, ethyl alcohol; insoluble in ether, xylene, benzene, chloroform, petroleum ether, etc.
Specific rotation in water	24°
Specific rotation in methyl alcohol	48°
Absorption maxima in water	265 mμ small band between 250 and 400 mμ
Oxidation-reduction potential, E_0^1	0.166 v. at pH 4.0 and 35°

The physical constants given by the United States Pharmacopeia are as follows:[4]

Solubility	1 g. dissolves in about 3 ml. of water and 30 ml. of alcohol
Specific rotation $[\alpha]_D^{25}$	In a solution containing the equivalent of 10 g. in 100 ml. of solution using a 200-mm. tube, between +20.5° and +21.5°

The following constants and method of assay are given by the British Pharmacopoeia:[5]

Molecular weight	176.1
Melting point	190° to 192° with decomposition
Specific rotation	Determined in a 2% w/v solution in water, +22° to +23°
	In 2% w/v solution in methyl alcohol, +50° to +51°
	In 2% w/v solution in a mixture of 12 ml. *N* sodium hydroxide and water to 100 ml., +112° to +115°
Ultraviolet absorption	Determined in a 0.002% w/v solution in water at pH 3 or less, at 245 mμ, 550

[3] P. György, Vitamin Methods, Vol. 2, p. 244. Academic Press, New York, 1951.

[4] United States Pharmacopeia, 14th revision, p. 57. Mack Printing Co., Easton, Pa., 1948.

[5] The General Medical Council, The British Pharmacopoeia 1948, p. 20. Constable and Co., London, 1948.

Assay

Dissolve about 40 mg., accurately weighed, in a mixture of 5 ml. of *water* and 5 ml. of *dilute sulfuric acid*, and titrate with 0.01 *N iodine*, using *mucilage of starch* as indicator. Each milliliter of 0.01 *N iodine* is equivalent to 0.0008805 g. of $C_6H_8O_6$.

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VII. Occurrence in Food

MAMIE OLLIVER

Investigations into the occurrence of ascorbic acid in foodstuffs have been facilitated by the comparative simplicity of chemical methods for estimation of the vitamin. Unfortunately, however, failure to observe essential precautions when applying such methods (see p. 247) has resulted, in some instances, in the publication of experimental data of doubtful value. Consequently, discrimination must be used when interpreting the conclusions from such work. Nevertheless, a large amount of reliable information relating to the antiscorbutic value of foods is available in the literature, and, indeed, the sources of information are so numerous that it has been found impracticable in the present review to attempt to present a comprehensive list of references.

A. UNTREATED FOODS

Consideration of the dietetic value of any food must first be directed to the raw material, and, in the case of vitamin C, this is virtually limited to plant tissues, milk, and liver, the other animal tissues used for foods being of relatively little practical importance. Raw liver contains about 30 mg. per 100 g. of ascorbic acid, and liquid milk, as supplied to the consumer, between 13 and 17 mg. per liter. At the present time, synthetic ascorbic acid may be added to some foods either for the purpose of fortification or as a processing aid, e.g., as an antioxidant, and the possibility of such additions should therefore also be borne in mind in dietary assays.

The distribution of ascorbic acid within one individual fruit or vegetable is often extremely variable. For instance, the vitamin has been found to be more concentrated in the skins than in the pulp of fruit; the leaves of spinach contain more ascorbic acid than the petioles; and the leaves from a single cabbage may show significantly different antiscorbutic values from one another. Similarly, individual cabbages of the same variety growing adjacent to one another in the same field may show different average concentrations of ascorbic acid. Berries on one plant may show similar variation, but this may be partially due to differences in degree of maturity,

which is known to be an important factor in determining the concentration of ascorbic acid in fruits and seeds. Thus, for instance, garden peas in the early stages of development have been found to contain as much as 160 mg. of ascorbic acid per 100 g., although when they are ready for harvesting the value falls to approximately 25 mg. per 100 g. Immature walnuts have been found to contain between 1500 and 2500 mg. of ascorbic acid per 100 g., although there is a steady decline as lignification takes place until the hardened nut has a negligible antiscorbutic value. With some vegetables, however, notably storage organs, such as potatoes, and leaves, such as spinach, the concentration of ascorbic acid has been found to remain relatively constant during the whole of the growth period.

In some fruits and vegetables, variety is important in determining the concentration of ascorbic acid in the tissues, but in others varietal effect is negligible compared with other factors. Manurial conditions may possibly also affect the amount of ascorbic acid, and some soil nutrients, such as molybdenum, have been found to increase the concentration of the vitamin in plants. Climatic conditions may also have an effect, and in localities where the incidence of sunshine is high there appears to be a corresponding increase in average ascorbic acid content. Seasonal variations may, however, occur which are not obviously related to climatic conditions. For instance, a laboratory in England made a survey of the ascorbic acid value of freshly picked black currants between 1936 and 1946. The results of testing many hundreds of samples of many varieties of this fruit from different localities showed relatively consistent results for the yearly average with the exception of 1941. In this year, the average concentration fell to 138 mg. per 100 g. as against 200 to 230 mg. per 100 g. for the other periods under review.

Nevertheless, in spite of the wide limits within which the ascorbic acid content of any fruit or vegetable might be expected to vary, the average figures quoted by various investigators do in fact show reasonably good agreement. For this reason, the compiling of a table showing such data has seemed justifiable (Table V), but the possibility of significant variation from these figures for individual batches must be appreciated. The literature has been searched to make this table as comprehensive as possible, but reference may be made to some sources which have been found of special value.[1-14] Only those plant organs which are normally used for food

[1] L. J. Harris and M. Olliver, *Biochem. J.* **36**, 155 (1942).

[2] M. Olliver, *Chemistry and Industry* **62**, 146 (1943).

[3] B. S. Platt, Tables of Representative Values of Foods Commonly Used in Tropical Countries, *Med. Research Council (Brit.), Spec. Rept. Ser.* **253** (1945).

[4] Nutritive Values of Wartime Foods, *Med. Research Council (Brit.) War Mem.* **14** (1945).

[5] H. E. Munsell, L. O. Williams, L. P. Guild, C. B. Troescher, G. Nightingale, and R. S. Harris, *Food Research* **14**, 144 (1949).

TABLE V

THE ASCORBIC ACID CONTENT OF FRESHLY HARVESTED FRUITS AND VEGETABLES
(Typical average values)

Food	Mg./100 g.	Food	Mg./100 g.
Apple	5	Kohlrabi	70
Apricot	7	Leek	15
Artichoke, Globe	9	Lemon (pulp)	50
Artichoke, Jerusalem	7	Lettuce	15
Asparagus	40	Lime (pulp)	27
Avocado	15	Loganberry	30
Banana	10	Mango	50
Bean, broad	30	Marrow	5
Bean, green, snap, or string	20	Melon, cantaloupe	33
Bean, lima	35	Melon, honeydew	23
Bean, wax or yellow	19	Melon, water	6
Beet, greens	50	Mint	35
Beetroot	6	Mulberry	10
Blackberry	20	Mushroom	3
Blueberry (Bilberry)	15	Okra	30
Broccoli, greens	120	Onion, mature	10
Broccoli, white head	70	Onion, spring	25
Brussels sprouts	100	Orange, sweet (pulp)	50
Cabbage	60	Orange, bitter (pulp)	35
Carrot	9	Orange, tangerine (pulp)	25
Cassava	35	Papaya	50
Cauliflower, white head	75	Parsley	190
Celery	9	Parsnip	15
Chard	38	Pea	25
Cherry	8	Peach	10
Coconut	2	Pear	4
Cowpea	30	Pepper, green	120
Cranberry	12	Persimmon	11
Cress, garden	80	Pineapple	25
Cucumber	10	Plantain	14
Currant, black	210	Plum	3
Currant, red	38	Pomegranate	6
Currant, white	40	Potato	30
Custard apple	10	Pumpkin	10
Damson	3	Quince	15
Date	0	Radish	27
Eggplant	8	Raspberry	25
Endive	15	Rhubarb	10
Fig	2	Spinach	60
Garlic	8	Squash, summer	17
Gooseberry	37	Squash, winter	10
Gourd	10	Strawberry	60
Grape	4	Swede (rutabaga)	35
Grapefruit (pulp)	40	Sweet corn	12
Greengage	5	Sweet potato	25
Granadilla (Passion fruit)	15	Tomato	25
Guava	300	Turnip	30
Horseradish	110	Turnip, greens	120
Kale	140	Watercress	60
		Yam	9

in Europe and the United States of America have been included. Rose hips (species of which have been found to contain more than 1000 mg. of ascorbic acid per 100 g.) have, however, been used in the diet in the form of sirup, and some Asiatic plants are of considerable interest in view of their reputed high vitamin C content. All fully mature nuts are negligible sources of the vitamin and have therefore been omitted from Table V.

1. Storage of Untreated Foods

Post-harvest storage may have a significant effect on the antiscorbutic value of the raw fruit or vegetable. The degree to which the vitamin is affected is associated with the time and temperature of storage, the extent to which the cellular tissue may be damaged after harvesting, the structure of the fruit or vegetable, and the presence or absence of ascorbic acid oxidase in the tissue. Hard fruit, such as pears and apples and citrus fruit, retain their vitamin C content over normal periods of storage, provided that the temperature is not allowed to rise unduly. Most soft berry fruits, however, especially when fully or overripe, show a rapid deterioration in quality after harvesting, and this is associated with a corresponding fall in ascorbic acid content. Firm berries such as gooseberries, however, can be kept for several days at room temperature without any appreciable change in vitamin C content. With root vegetables, long-term storage is to be anticipated and, in such cases, a progressive fall in vitamin C content is to be expected (Table VI). Green vegetables, on account of wilting, can be held at room temperature for a maximum of only a few days after harvesting. The tightly packed leaves in a head of cabbage will show a negligible loss of vitamin, even when wilted, whereas the loose leaves of spinach show a rapid loss under similar conditions. Low-temperature storage in all cases slows down the rate at which the ascorbic acid content is decreased.

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[6] B. K. Watt and A. L. Merrill, *U.S. Dept. Agr., Agr. Handb.* **8** (1950).

[7] H. E. Munsell, L. O. Williams, L. P. Guild, C. B. Troescher, G. Nightingale, and R. S. Harris, *Food Research* **15,** 16 (1950).

[8] H. E. Munsell, L. O. Williams, L. P. Guild, C. B. Troescher, G. Nightingale, and R. S. Harris, *Food Research* **15,** 34 (1950).

[9] H. E. Munsell, L. O. Williams, L. P. Guild, C. B. Troescher, G. Nightingale, L. T. Kelley, and R. S. Harris, *Food Research* **15,** 263 (1950).

[10] H. E. Munsell, L. O. Williams, L. P. Guild, C. B. Troescher, and R. S. Harris, *Food Research* **15,** 355 (1950).

[11] H. E. Munsell, L. O. Williams, L. P. Guild, L. T. Kelley, A. M. McNally, and R. S. Harris, *Food Research* **15,** 379 (1950).

[12] H. E. Munsell, L. O. Williams, L. P. Guild, L. T. Kelley, and R. S. Harris, *Food Research* **15,** 421 (1950).

[13] H. E. Munsell, L. O. Williams, L. P. Guild, L. T. Kelley, A. M. McNally, and R. S. Harris, *Food Research* **15,** 439 (1950).

[14] Wu, L. Woot-Tsuen, R. K. Pecot, and B. K. Watt, *U.S. Dept. Agr., Agr. Handb.* **34** (1952).

B. PROCESSED FOODS

When assessing the dietetic value of any foodstuff, consideration should be given to the form in which it is likely to reach the consumer, and the figures given in Table V may therefore be misleading unless they are considered in relation to the effect of different methods of preparation. The high solubility of ascorbic acid in water and the relative ease with which it is oxidized, either chemically or enzymatically, make this vitamin particularly susceptible to processing conditions, especially when the tissue is broken or when the temperature is raised. Slicing, cutting, or bruising of fruits and vegetables before processing is therefore likely to result in loss of ascorbic acid, especially when associated with high-temperature treatment

TABLE VI

AVERAGE ASCORBIC ACID CONTENT OF POTATOES AT DIFFERENT TIMES OF THE YEAR[a]

Month	Average ascorbic acid content, mg./100 g.
August[b]	35
September[b]	35
October	23
November	19
December	16
January	12
February	10
March and onward	6

[a] Taken from table by M. Olliver from "Cooking and Nutritive Value," A. B. Callow, Oxford Clarendon Press, 1945.

[b] The values for August and September were obtained from freshly dug potatoes, and for October onward from stored potatoes.

in air, water, or steam. Cold-water washing or steeping does not normally leach out any significant amount of the vitamin, provided that the tissue is not unduly broken.

1. BOILING, STEAMING, AND PRESSURE COOKING

Investigations into household methods of cooking have shown that, when fruits or vegetables are boiled with water, the ascorbic acid is relatively stable. Significant losses due to oxidation are usually found only in large-scale catering where the bulk addition of vegetables to the cooking water may result in lowering of the temperature to such a degree that the action of the enzymes is temporarily accelerated. Extraction of the vitamin does, however, take place rapidly under normal conditions of boiling and continues until the concentration of vitamin in the liquor approximates to that in the tissue. This point is usually reached after the optimum pala-

tability of the vegetables has been passed, and extended cooking should therefore be avoided. Similarly, a relatively higher loss is to be anticipated as the ratio of water to vegetable is increased. From the dietetic point of view, this leaching is of little significance with stewed fruits, where the juice is eaten with the tissue, but it may represent a serious loss with vegetables when the cooking water is drained away before serving.

If vegetables are steamed instead of boiled, the leaching effect is negligible but a greater loss of vitamin is to be expected from oxidation. In pressure cooking, owing to the extremely small amount of water used, the amount of ascorbic acid lost by extraction is small but, compared with

TABLE VII

EFFECT OF HOUSEHOLD COOKING ON ASCORBIC ACID CONTENT OF VEGETABLES
(Typical values)

Method	% Vitamin C		
	Destroyed	Extracted	Retained
Green vegetables			
Boiling A[a]	10–15	45–60	25–45
Boiling B[b]	10–15	15–30	55–75
Steaming	30–40	<10	60–70
Pressure cooking	20–40	<10	60–80
Root vegetables[c]			
Boiling	10–20	15–25	55–75
Steaming	30–50	<10	50–70
Pressure cooking	45–55	<10	45–55

[a] Ratio of water to vegetable high; long cooking time.
[b] Ratio of water to vegetable low; short cooking time.
[c] Whole or large pieces.

normal boiling, the destruction is greater. The over-all effect is that identical vegetables cooked under increased pressure and at normal pressure have similar antiscorbutic values, provided that the processing is continued in each case only to the point of maximum palatability of the vegetable. Extended time of cooking will result in correspondingly greater loss of the vitamin when pressure is used than with normal boiling methods.

Keeping drained vegetables hot for an extended period before serving is to be deprecated from the nutritional standpoint since progressive oxidation of the vitamin has been found, especially when the tissue is finely cut or divided as with mashed potatoes or chopped green vegetables.

In Table VII attempts have been made to summarize the general findings of many workers when different methods of cooking have been investigated. It will be appreciated, however, that, owing to the number of possible

variants involved, these figures can be accepted as indicative of results only for average and controlled conditions.

2. Jam (Preserves)

When fruit is boiled with sugar, as in jam making, the ascorbic acid is remarkably stable, although a significant loss from the original fruit may be entailed if the fruit is soft and broken or pulped or cut and allowed to stand before boiling. The most important factor controlling the vitamin C content of the finished jam is, indeed, the condition of the fruit used for preservation. The rate at which ascorbic acid is destroyed on storage of the jam is associated with temperature of storage but, under normal conditions, only slight loss is to be predicted over several weeks.

3. Freezing

The blanching or scalding process given to vegetables which are to be commercially frozen, dehydrated, or canned consists in treating the cleaned, trimmed material at a high temperature for a few minutes in order to inactivate the enzymes. If this procedure is omitted, then off-flavors, discoloration, and loss of ascorbic acid may occur during subsequent processing operations and, with frozen and dehydrated vegetables, during post-processing storage. If hot-water blanching is used, ascorbic acid may be leached from the plant tissues, whereas with steam blanching oxidation of the vitamin may occur. To minimize these losses, various precautions are normally taken which include short time-high temperature treatment, serial blanching (whereby successive batches of fresh vegetables are passed through the same scalding liquor), and the use of sulfite.

In the preparation of frozen foods, the actual freezing process appears to have a negligible effect on ascorbic acid content, but post-freezing storage is important and a temperature of not higher than $-20°$ F. has been found necessary in order to retain the maximum content of the vitamin for 10 months' storage. Thawing of the food before cooking may result in progressive loss of the vitamin, especially if enzymes are present, and indeed it is one of the most important factors affecting the ascorbic acid content of the food as eaten.

By reason of the partial softening of the tissues during blanching, the final product needs proportionately shorter cooking time and also less water than when the raw material is cooked. Consequently, when commercially frozen vegetables are given a final household cooking, there is significantly greater retention of ascorbic acid than with the heating of fresh vegetables. This normally offsets the loss of the vitamin which occurs during controlled blanching and results in similar antiscorbutic values for the fresh and processed material when prepared for the table.

4. Dehydration

The loss of ascorbic acid in controlled modern methods of dehydration, whereby the blanched tissue is rapidly dried, is small compared with the older drying processes which destroyed most of the vitamin. Some oxidation does take place, however, and this, in conjunction with blanching loss, may mean that, for instance, in cabbage only some 50 to 60 % of the original ascorbic acid is retained. However, since no further loss is to be anticipated on storage of the dehydrated tissue in nitrogen over a period of several months and since reconstitution again brings about no further degradation of the vitamin, the product as eaten is likely to be of only slightly less antiscorbutic value than freshly harvested material after household cooking.

5. Canning and Bottling

During the canning or bottling of fruits and vegetables, there is very little destruction of ascorbic acid, but leaching into the liquor takes place. On account of the relatively lower ratio of liquid to solid than in household saucepan cooking, less vitamin is extracted and therefore less loss occurs when the vegetable liquor is discarded. Destruction of ascorbic acid gradually takes place on storage of the processed material, the rate of loss being accelerated with increasing headspace, by high-temperature storage, and, when jars are used, by the action of light. With vegetables, these storage losses have to be considered alongside losses due to blanching. In general, however, the total loss is usually offset by the stability of the ascorbic acid during the reheating process. Consequently, canned fruits and vegetables which have been stored for a few months under normal conditions may be accepted as having a comparable antiscorbutic value in the diet as similar material prepared by open saucepan cooking.

6. Assessment of Ascorbic Acid Content of Diets

Unless conditions of processing of the foodstuff are known, it is almost impossible to predict the vitamin C content of a diet from a knowledge of the fruits and vegetables consumed, and chemical assays must be used. If information about such processing, and especially the final household treatment, is known, however, then over a period of several weeks it is possible for the average daily intake of ascorbic acid to be determined, with a reasonable degree of accuracy, from a knowledge of the quantities of different fruits and vegetables which have been eaten. Household cooking is likely to prove not only the most important but also the most unpredictable factor in determining the antiscorbutic value of the diet, and any assessment by calculation should always be substantiated by chemical assay of a proportion of the individual components of the diet.

VIII. Effects of Deficiency in Animals

MARY ELIZABETH REID

A. OUTSTANDING DISCOVERIES

The following discoveries constitute milestones marking progress in our knowledge of the nutritional aspects of ascorbic acid. Many of the findings are concerned with the development of new methods of approach to critical problems in the fields of physiology, morphology, pathology, and biochemistry.

1536 — Cartier[1] while exploring the St. Lawrence River saved many of his crew from scurvy by feeding them an infusion made from pine needles.

1753 — Lind[2] established that scurvy can be prevented or cured by fresh fruits or vegetables in the diet.

1907 — Holst and Fröhlich[3] produced experimental scurvy in the guinea pig.

1915 — Ingier[4] showed experimentally the effects of scurvy on pregnancy and on the young.

1919 — Zilva and Wells[5] showed that the teeth are among the first, if not the first, organs to be affected by the deficiency.

1920 — Parsons[6] presented convincing evidence that the rat can synthesize the vitamin.

1924 — Höjer[7] demonstrated that microscopic changes in the teeth of guinea pigs could be used as a method of assaying for the antiscorbutic substance.

1926 — Wolbach and Howe[8] presented experimental evidence that scurvy in guinea pigs is associated with failure in the formation of intercellular substance.

1928 — Szent-Györgyi[9] isolated a "hexuronic" acid from adrenals.

1930 — Dalldorf and Zall[10] showed that the rate of growth of the incisor teeth of guinea pigs is closely related to the vitamin C intake.

[1] H. P. Biggar, The Voyages of Jacques Cartier. Publications of Public Archives of Canada, No. 11, 1924.

[2] J. Lind, Treatise on Scurvy, 2nd ed. A. Millar, London, 1757.

[3] A. Holst and T. Fröhlich, *J. Hyg.* **7**, 634 (1907).

[4] A. Ingier, *J. Exptl. Med.* **21**, 525 (1915).

[5] S. S. Zilva and F. M. Wells, *Proc. Roy. Soc.* (*London*) **B90**, 505 (1919).

[6] H. T. Parsons, *J. Biol. Chem.* **44**, 587 (1920).

[7] J. A. Höjer, *Acta Paediat. Suppl.* **3**, 8 (1924).

[8] S. B. Wolbach and P. R. Howe, *Arch. Pathol.* **1**, 1 (1926).

[9] A. Szent-Györgyi, *Biochem. J.* **22**, 1387 (1928).

[10] G. Dalldorf and C. Zall, *J. Exptl. Med.* **52**, 57 (1930).

1930, 1932 — Tillmans and associates[11] demonstrated that the indicator dichlorophenolindophenol could be used to measure the antiscorbutic potency of foods.

1932 — Szent-Györgyi and Haworth[12] and Waugh and King[13] identified the "hexuronic" acid as vitamin C.

1933 — Haworth[14] determined the structure of the ascorbic acid molecule.

1933 — Reichstein, Grüssner, and Oppenauer[15] synthesized ascorbic acid.

1936 — Stephens and Hawley[16] demonstrated the high content of the vitamin in the leucocytes.

1939 — Sealock and associates[17] demonstrated that ascorbic acid is concerned in the metabolism of tyrosine.

1943, 1948 — Roe and associates[18, 19] developed the osazone method of assaying for the vitamin.

1944 — Crampton and associates[20] demonstrated the "growth of the odontoblasts" method of assay.

1947 — Nungester and Ames[21] showed convincingly the relationship between ascorbic acid and phagocytic activity.

1948 — Christensen and Lynch[22] showed that a marked decrease in glycine and glutamine and an increase in other amino acids occurs in scorbutic muscles.

1950, 1951 — King and associates[23, 24] demonstrated in studies with radioactive ascorbic acid that much of the administered vitamin is broken down to carbon dioxide.

B. GROSS EFFECTS OF DEFICIENCY

Scurvy is a disease resulting from severe ascorbic acid deficiency. Only the primates and guinea pigs are susceptible to it, because only they lack

[11] J. Tillmans, P. Hirsch, and W. Hirsch, *Z. Untersuch. Lebensm.* **60,** 34 (1930); **63,** 1 (1932).

[12] A. Szent-Györgyi and W. N. Haworth, *Nature* **131,** 24 (1933).

[13] W. A. Waugh and C. G. King, *J. Biol. Chem.* **97,** 325 (1932).

[14] W. N. Haworth, *Chemistry & Industry* **53,** 482 (1933).

[15] T. Reichstein, A. Grüssner, and R. Oppenhauer, *Helv. Chim. Acta* **16,** 1019 (1933).

[16] D. J. Stephens and E. E. Hawley, *J. Biol. Chem.* **115,** 653 (1936).

[17] R. R. Sealock, B. Ziegler, and R. L. Driver, *J. Biol. Chem.* **128,** lxxxix (1939).

[18] J. H. Roe and C. A. Kuether, *J. Biol. Chem.* **147,** 399 (1943).

[19] J. H. Roe, M. B. Mills, M. J. Oesterling, and C. M. Damron, *J. Biol. Chem.* **174,** 201 (1948).

[20] E. W. Crampton, B. C. Collier, L. D. Woolsey, and F. A. Farmer, *Science* **100,** 599 (1944).

[21] W. J. Nungester and A. M. Ames, *J. Infectious Diseases* **83,** 50 (1948).

[22] H. N. Christensen and E. L. Lynch, *J. Biol. Chem.* **172,** 107 (1948).

[23] J. J. Burns, H. B. Burch, and C. G. King, *J. Biol. Chem.*, **191,** 501 (1951).

[24] S. S. Jackel, E. N. Mosbach, J. J. Burns, and C. G. King, *J. Biol. Chem.* **186,** 569 (1950).

the capacity to synthesize the vitamin. The guinea pig has been the subject of most of the experimental studies on the physiological and pathological aspects of ascorbic acid deficiency. If young animals weighing approximately 300 g. are placed on a diet completely devoid of ascorbic acid but adequate in all other respects, they usually show a slight loss in weight during the first few days because of the change in type of diet. This is followed by a period of growth which usually continues until about the tenth to sixteenth day, after which there is a rapid loss in weight associated with

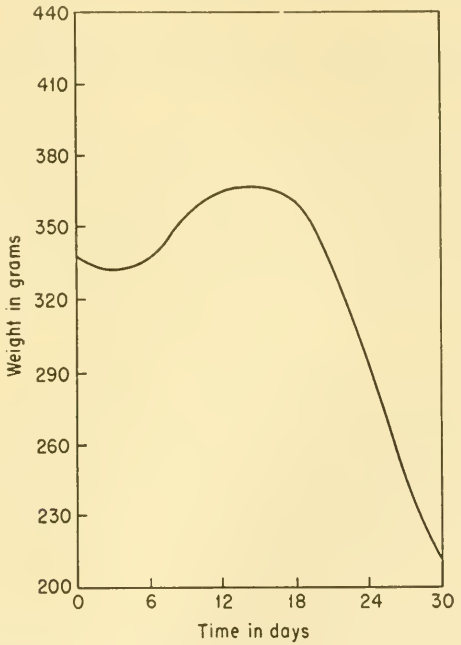

FIG. 1. Average weight curves of guinea pigs on a scorbutigenic diet. (From Sherman and Smith.[26])

the onset of the first symptoms of scurvy. Death usually occurs from about the twenty-fifth to the twenty-eighth day, slightly later if the initial weight of the animals was over 300 g. and earlier if appreciably under 300 g.

Figure 1 shows the curve representing the average weights of 10 guinea pigs as obtained by Sherman *et al.*,[25, 26] using the scorbutigenic diet which they developed.

The outstanding symptoms of scurvy in the guinea pig are hemorrhage

[25] H. C. Sherman, V. K. LaMer, and H. L. Campbell, *J. Am. Chem. Soc.* **44**, 165 (1922).

[26] H. C. Sherman and S. L. Smith, The Vitamins, p. 171. Chemical Catalog Co., New York, 1931.

in almost any part of the body, particularly in intramuscular and subcutaneous areas, and a general weakness of tissues, especially in those with a comparatively high content of collagen or related substances. Other characteristic symptoms are loss of appetite, lessening of activity, loss of luster of eyes and hair, roughening of hair, and assumption of "face-ache" posture—hunching with drooping head, stiffening of hind legs and frequent outward rotation of legs, beading of ribs—and in the late stages there is usually a lowering of body temperature, anemia, increase in leucocytes, and decrease in amount of blood, especially near the surface, and a tendency to diarrhea.

Certain symptoms, which have been described as characteristic of scurvy in guinea pigs (by some but not all of the investigators in this field), may possibly be due to some associated deficiency. Included in this category are much swollen joints and the presence of large amounts of fluid in the body cavity. The swollen gums and loose teeth which are characteristic of scurvy in primates do not occur in guinea pigs under the usual experimental conditions but only when the diet contains enough ascorbic acid to markedly prolong life, thereby allowing sufficient time for the symptoms to develop.

C. PATHOLOGY

1. Mesenchymal Tissues

The most notable effects of ascorbic acid deficiency are to be found in the mesenchymal tissues, but other tissues are also affected.[27]

According to Schade[28] the mesenchyme has three general functions: (1) supporting—holding the parenchyma cells of the organs in correct relation; (2) filling in the complementary spaces of the body which constantly change in form with the movement of the muscles; and (3) gliding—permitting the smoothest possible shifting of the different parts. Meyer[29] has suggested another function of connective tissue, i.e., a probable chemical cooperation between the stroma and epithelium in all epithelial and endothelial structures.

Aschoff and Koch[30] were the first investigators to suggest that the primary deficiency in scurvy is due to a lack, or a faulty development, of intercellular cement materials. Höjer,[7] on the other hand, held that the disturbance was directly due to a functional defect of the cells rather than to a lack of structural materials. Regardless of which of these concepts is correct,

[27] S. B. Wolbach, *New Engl. J. Med.* **215,** 1158 (1936).

[28] H. Schade, Die Molekularpathologie der Entzündung. Theodor Steinkopff, Dresden and Leipzig, 1935.

[29] K. Meyer, *Trans. 1st Conf. on Connective Tissues, New York* (1950).

[30] L. Aschoff and W. Koch, Eine Pathologische-Anatomische Studie. Gustav Fischer Jena, 1919.

when the vitamin is not supplied, the cells are unable to produce intercellular substances such as collagen, ossein, and dentine. The supporting tissues are particularly affected, especially those in which the intercellular substance is calcified. Cell divisions may proceed at almost a normal rate, at least in the early stages of the deficiency, but the cells fail to mature and differentiate and because of this they are unable to perform their special functions. Glazunow[31] considers this failure in differentiation to be the primary defect in scurvy. Associated with this condition of immaturity is a disorderly arrangement of the cells, a lack of proper orientation. Possibly the cause of these failures lies primarily in a disorderly arrangement of molecules in the cytoplasm, particularly at the surface of the cell.

a. Connective Tissue

Connective tissue is made up of fibers secreted by the fibroblasts[32, 33] and embedded in an amorphous matrix which may or may not be secreted by cells other than the fibroblasts.[34, 35] Collagen is the chief but not the only constituent of the fibers. It and its allied substances serve as framework materials for animals much like cellulose, and its allied substances make up the structural elements of plant tissues. Both types of framework substances are characterized by sensitivity to alkali with the production of acidic groups and fission of the chains of longitudinally oriented molecules, polypeptides in animal fibers and glucose groups in plant fibers. There is evidence from x-ray studies, though not conclusive, that in both types of fibers the regularly arranged units are held together by side-chain linkages.[36, 37] Also, in each type of fiber the regularly arranged units are surrounded by non-crystalline interfibrillar material of which carbohydrates and uronic acids are characteristic constituents. By secondary linkages with the basic fiber constituents, these carbohydrate-uronic acid groups could presumably serve as gelling agents in both types of tissue. It has been shown[38] that the cell walls of embryos in guinea pigs maintained on an ascorbic acid-deficient diet tend to be indistinct and blurred in appearance in contrast to well-defined cell walls in corresponding tissues of the control animals. A feature suggesting a chemical relationship in the cell framework structures of both plants and animals is that crude pectinase solutions will dissolve the cementing material in both groups. If this

[31] M. Glazunow, *Virchow's Arch. pathol. Anat. u. Physiol.* **299**, 120 (1937).
[32] M. L. Stearns, *Am. J. Anat.* **66**, 133; **67**, 55 (1940).
[33] K. R. Porter, *Trans. 2nd Conf. on Connective Tissues, New York* (1951).
[34] M. Staemmler, *Frankfurt. Z. Pathol.* **25**, 391 (1921).
[35] B. Sylvén, *Acta Chir. Scand.* **86**, *Suppl.* 66 (1941).
[36] F. O. Schmitt, *Physiol. Revs.* **19**, 270 (1939).
[37] W. N. Haworth, *Chemistry & Industry* **58**, 917 (1939).
[38] M. T. Harman and L. E. Warren, *Trans. Kansas Acad. Sci.* **54**, 42 (1951).

reaction should occur with pure preparations of the enzyme, it would be of considerable interest.[39, 40]

(1) *Fibers.* The relative proportion of the total nitrogen of the body found in collagen is shown in a study made by Lightfoot and Coolidge[41] on the distribution of collagen in a half-grown guinea pig. In the hairless animal the collagen nitrogen constituted 28.6% of the total nitrogen. The fascia contained a large portion of the total collagen. In similar studies with rats Neuberger *et al.*[42] estimated that about one-third of the total mass of the protein of the body consists of collagen.

Many of the original mesenchymal cells of the embryo become transformed into fibroblasts. The role of these cells and the stimulating effect upon them of ascorbic acid in the formation of connective tissue fibers has been developed and elucidated largely from studies made with tissue cultures, inflammatory reactions, and in healing experimentally induced wounds. Von Jeney and Törö[43] grew fibroblasts in tissue cultures and found that addition of ascorbic acid caused a more rapid production of fibers. Mazoué[44] and Querido and Gaillard[45] confirmed this observation. Wolbach and Howe[8] showed that when incisions were made in the skin of scorbutic animals the wounds failed to heal. After experimental bone injuries, new bone formation failed to take place. Proliferation of the fibroblasts, osteoblasts, epidermis, and endothelium was almost as active as in the normal animals. However, the fibroblasts and osteoblasts were unable to differentiate and mature. The results of other studies of wound healing and inflammatory reactions have confirmed and added to these findings.[27, 44, 46-52] Hunt found that in normal guinea pigs a mature vascular scar was formed in 14 days.[53] Bartlett *et al.*[51] have shown that ascorbic acid apparently accumulates in and around traumatized tissues. This increase presumably may be a factor in the accumulation of fibroblasts and

[39] J. F. A. McManus and J. C. Saunders, *Science* **111**, 204 (1950).
[40] J. F. A. McManus and J. E. Cason, *Arch. Biochem. and Biophys.* **34**, 293 (1951).
[41] L. H. Lightfoot and T. B. Coolidge, *J. Biol. Chem.* **176**, 477 (1948).
[42] A. Neuberger, J. C. Perrone, and H. G. B. Slack, *Biochem. J.* **49**, 199 (1951).
[43] A. Von Jeney and E. Törö, *Virchow's Arch. pathol. Anat. u. Physiol.* **298**, 87 (1936).
[44] H. Mazoué, *Compt. rend. soc. biol.* **126**, 991 (1937).
[45] A. Querido and P. J. Gaillard, *Acta Brevia Neerl. Physiol. Pharmacol. Microbiol.* **9**, 70 (1939).
[46] T. H. Lanham and T. H. Ingalls, *Ann. Surg.* **105**, 616 (1937).
[47] M. Taffel and S. C. Harvey, *Proc. Soc. Exptl. Biol. Med.* **38**, 518 (1938).
[48] H. J. Lauber and W. Rosenfeld, *Klin. Wochschr.* **17**, 1587 (1938).
[49] G. Bourne, *Lancet* **243**, 661 (1942).
[50] J. B. Hartzell and W. E. Stone, *Surg. Gynecol. Obstet.* **75**, 1 (1942).
[51] M. K. Bartlett, C. M. Jones, and A. E. Ryan, *Ann. Surg.* **111**, 1 (1940).
[52] E. M. Meyer and M. B. Meyer, *Bull. Johns Hopkins Hosp.* **74**, 98 (1944).
[53] A. H. Hunt, *Brit. J. Surg.* **28**, 436 (1941).

leucocytes in the area. Moreover, their prompt absorption of the vitamin may make possible its continued inflow into the area. Dalldorf[54] describes the action of the vitamin in wound healing under normal conditions as follows: "Under certain conditions the type cell, the fibroblast, lies in an amorphous ground substance within which fibrils (reticulin) are formed which may in turn become gathered into wavy bands of collagen. In this transformation the fibrils seem to become cemented together by a translucent matrix, the formation suggesting a colloid phenomenon, the setting of a gel."

Several investigators have shown that the strength of a scar is dependent on the intercellular substances laid down by the fibroblasts and that within certain limits a quantitative relation exists between the amount of ascorbic acid available and the amount of intercellular materials produced, though increasing the intake above the normal daily requirements does not produce greater than normal tensile strength of wounds.[48, 50] Danielli *et al.*[55] found that with doses of less than 2 mg. of ascorbic acid per day large amounts of reticulin were formed, although the appearance of the wound was not normal. Different types of tissue have different ascorbic acid requirements. Galloway *et al.*[56] found that the healing of wounds in skin and cartilage was much influenced by the ascorbic acid nutrition whereas the need of the vitamin for healing wounds in epithelial tissue appeared to be much less. Wolbach's studies[27] had also indicated this.

Hunt[53] reported that in scurvy the intercellular material was fluid and amorphous. In newly formed granulation tissue of an otherwise normal animal there is an abundance of metachromatic staining material which becomes less as the scar matures[27, 49, 54, 57, 58] but with lack of ascorbic acid persists and, moreover, remains in a fluid or semifluid state. Wolbach[59] suggested that this fluid might have originated as a discharge from the vacuoles which he observed in the fibroblasts, particularly at their extremities. Recent studies[57, 60] have indicated, however, that fat is present in the vacuoles. Within a few days after administration of ascorbic acid the intercellular material shows an increase in reticulin fibers, stainable with silver nitrate. As they mature to collagen the fibers appear to lose their ability to blacken in silver and develop an affinity for Van Giesen's stain.[53, 59] Hunt[53] reported that in scurvy the transformation of reticulin to collagen is retarded or stops entirely, depending on the severity of the

[54] G. Dalldorf, *J. Am. Med. Assoc.* **111**, 1376 (1938).
[55] J. F. Danielli, H. B. Fell, and E. Kodicek, *Brit. J. Exptl. Pathol.* **26**, 367 (1945).
[56] N. M. Galloway, R. C. Garry, and A. D. Hitchins, *Brit. J. Nutrition* **2**, 228 (1948).
[57] J. R. Penney and B. M. Balfour, *J. Pathol. Bacteriol.* **61**, 171 (1949).
[58] H. Bunting, *Ann. N. Y. Acad. Sci.* **52**, 977 (1950).
[59] S. B. Wolbach, *Am. J. Pathol.* **9**, 689 (1933).
[60] P. Klemperer, *Am. J. Pathol.* **26**, 505 (1950).

deficiency. However, the more recent view, derived from x-ray studies, is that, other than in size, there is no real structural difference between reticulin and collagen.[33, 61, 62]

(a) Studies on the Ascorbic Acid Maintenance Requirement of Normal Collagen. Hunt[53] stated that mature collagen in a scar may retrogress if ascorbic acid is subsequently withdrawn from the diet and suggested that the ready reversion of the collagen of scars to its immature form affords an explanation of the breaking down of healed wounds in ascorbic acid deficiency. This observation is in line with the report[63] that reopening of old wounds often occurred in British sailors at times when scurvy was common. Pirani and Levenson[64] found that ascorbic acid is necessary not only for the healing of wounds in guinea pigs but also for the maintenance of scar tissue which had formed in the wounds over a period of many weeks. Morrione[65] has recently presented evidence indicating that scar tissue can break down. Elster[66] studied the problem of the relation of ascorbic acid to the maintenance of collagen, using weanling guinea pigs with an average initial weight of 185 g. He made determinations of the collagen content of lungs, liver, kidneys, spleen, heart, and skeletal muscles of scorbutic animals with normal animals as age and weight controls. He found a lower content of collagen in lungs, liver, and kidneys in the scorbutic animals than in the controls of the same age but not lower than in the controls of the same weight. His results were interpreted as indicating that, "once formed, the fiber seems to be independent of further vitamin C nutrition except in so far as it is destroyed by the usual catabolic actions of the body and requires resynthesis."

The difficulty of separating and evaluating growth and maintenance requirements of ascorbic acid in relation to the collagen content of tissues is indeed very great, and undoubtedly the problem requires an approach from several different angles. Robertson[67] found no decrease in the collagen content of skin, liver, kidney, lung, or spleen during the development of acute or chronic scurvy but did find significant losses in teeth and costochondral junctions. Later,[68] he studied the effect of ascorbic acid deficiency on the collagen concentration of newly induced fibrous tissue produced by wounding, by subcutaneous injection of diacetyl phosphate or Irish moss extract, or by wrapping a kidney in plastic. He found that the collagen in

[61] J. Gross, *Ann. N. Y. Acad. Sci.* **52,** 964 (1950).
[62] J. H. Highberger, J. Gross, and F. O. Schmitt, *J. Am. Chem. Soc.* **72,** 3321 (1950).
[63] A. F. Hess, Scurvy—Past and Present. J. B. Lippincott Co., Philadelphia, 1920.
[64] C. L. Pirani and S. M. Levenson, *U. S. Army Med. Nutrition Lab. Rept.* **94** (1952).
[65] T. G. Morrione, *Trans. 2nd Conf. on Connective Tissue, New York*, p. 159 (1951).
[66] S. K. Elster, *J. Biol. Chem.* **186,** 105 (1950).
[67] W. V. B. Robertson, *J. Biol. Chem.* **187,** 673 (1950).
[68] W. V. B. Robertson, *J. Biol. Chem.* **196,** 403 (1952).

the healed wounds or new subcutaneous tissue was not decreased in concentration when the animals were made scorbutic. However, the collagen concentration of the new tissue about the kidney was decreased. His results led to the suggestion that the major portion of the newly induced collagen, as had been previously found with organ collagen, does not need ascorbic acid for maintenance.

Perrone and Slack[69] studied the relative metabolic inertia of collagen in the rat, using glycine labeled with C^{14} in the methylene group. Each rat received 50 mg. of glycine containing 20 μc. of radioactive glycine per 200 g. of body weight. Animals were killed after periods varying from 1 to 34 days. The extremely low radioactivity of the glycine isolated from the collagen indicated that the turnover rate of this protein is very slow, much more so than that of the mixed proteins of muscle. The results suggested the possibility that "the collagen is, in fact, completely inert and that the observed radioactivity is entirely due to the deposition of new collagen." (The animals were growing.) In later studies,[70] also with rats, these investigators determined the collagen in tendon, skin, bone, and liver in three groups of rats initially ranging in weight from 50 to 70 g., 200 to 240 g., and 350 to 370 g. Each rat received the C^{14} glycine in amounts equal to 10 μc. per 100 g. of body weight. Collagen was extracted, freed from other proteins, converted to gelatin, the glycine content determined, and the specific activity measured. The results indicated "some metabolic activity in collagen from skin, bone, tendon and liver during the first 3 weeks in young rats, very little activity in old rats at any time and variable metabolic activity in the 'young adult' group." Robertson[71] followed with results of an investigation of the influence of ascorbic acid on N^{15} incorporation into collagen in guinea pigs in which (a) the weight was maintained constant, (b) animals lost weight because of food restriction, (c) were acutely scorbutic, and (d) were recovering from acute scurvy. In general, the results showed that under conditions in which the food intake was low there was a low concentration of N^{15} in the collagen, regardless of the fact that a constant amount of the isotope had been fed. No specific effect of ascorbic acid was observable. The results were interpreted as suggesting that nitrogen turnover in collagen occurred without an appreciable breakdown of the macromolecule.

Results of the various investigations herein reported tend to indicate that the collagen turnover in adult tissues is extremely low. Although in scurvy there is considerable histological evidence of a partial breakdown of collagen in several types of tissue, the chemical methods of estimation do not reveal possible changes in its physical form.

[69] J. C. Perrone and H. G. B. Slack, *Biochem. J.* **48**, iv (1951).
[70] J. C. Perrone and H. G. B. Slack, *Biochem. J.* **49**, lxxii (1951).
[71] W. V. B. Robertson, *J. Biol. Chem.* **197**, 495 (1952).

(2) *Interfibrillar Substance.* To what extent ascorbic acid is concerned in the elaboration of the interfibrillar substances has not been conclusively determined. The interfibrillar substance has been shown to be in part composed of mucopolysaccharides, with hyaluronic acid and chondroitin sulfuric acids in their various forms constituting the main types which have been identified.[72, 73] Some of the chondroitin sulfuric acid esters of the interfibrillar substance are linked to protein, whereas the hyaluronic acid probably is not thus bound.

(a) Hyaluronic Acid. Hyaluronic acid is a complex substance found in some but not all types of connective tissue.[73] Meyer and associates[72] indicated that its basic unit is a disaccharide composed of acetylglucosamine and glucuronic acid with the main linkages glucosidic. Penney and Balfour[57] reported that normal guinea pigs elaborated mucopolysaccharides in the early stages of wound healing but there was a failure to produce them in the wounds of ascorbic acid-depleted animals. Injections of the vitamin into depleted animals resulted in the appearance of mucopolysaccharides in the wound within 12 hours. Even with small doses of the vitamin, large amounts of polysaccharides were formed. The first step leading to fiber formation appeared to be the deposition of these substances around the fibroblasts. These observations were confirmed by Klemperer and associates.[60] Pirani and Catchpole[74] reported a significant elevation in the level of serum glycoproteins in both acute and chronic scurvy in guinea pigs. However, there was not a close relation between the severity of the disease and the glycoprotein levels. After administration of ascorbic acid the level was reduced. No attempt was made to determine the nature of the carbohydrate involved in the polymerization changes. These investigators suggest that the high level of serum polysaccharides in scurvy may be a result of depolymerization of the carbohydrate-containing constituents of the ground substance and release of the carbohydrate moieties into the circulation. Bradfield and Kodicek[75] found that wound sections from normal and scorbutic guinea pigs differed markedly in their staining reactions. Those from the scorbutic animals contained numerous chaotically arranged precollagen fibers which stained intensely. In wounds from normal animals the PAS (periodic acid—Schiff) staining material diminished in the late stages of healing whereas in the scorbutic animals it increased. It formed a sheath around the non-staining precollagen fiber. The latter was removable by collagenase. Unlike Gersh and Catchpole's[76] findings, this mucopolysacchar-

[72] K. Meyer, *Ann. N. Y. Acad. Sci.* **52**, 961 (1950).
[73] K. Meyer and M. M. Rapport, *Science* **113**, 596 (1951).
[74] C. L. Pirani and H. R. Catchpole, *U. S. Army Med. Nutrition Lab. Rept.* **81** (1951).
[75] J. R. G. Bradfield and E. Kodicek, *Biochem. J.* **49**, xvii (1951).
[76] I. Gersh and H. R. Catchpole, *Am. J. Anat.* **85**, 457 (1949).

ide material was not markedly water-soluble and appeared not to be removable with hyaluronidase.

An effect of ascorbic acid, apparently opposite to that described by Pirani and Catchpole, has been convincingly demonstrated by several investigators.[77-83] A disaggregating effect of the vitamin on body fluids containing hyaluronic acid was first shown by Robertson *et al.*,[77, 78] who studied its influence on the viscosity of mucins from the vitreous body and synovial fluid. Skanse and Sunblad[82] obtained the same type of results with solutions of reportedly pure hyaluronic acid isolated from various sources. Traces of copper accelerated the disaggregating effect of ascorbic acid. The most interesting aspect of this line of investigation was the finding that ascorbic acid and hydrogen peroxide acting together produce a very rapid degradation effect. Hydrogen peroxide alone has also been found to have a depolymerizing action.[82, 83] Daubenmerkl[83] has shown that the effect of an equimolar mixture of ascorbic acid and hydrogen peroxide increases with rising concentrations, a result which is not found with ascorbic acid alone. His studies *in vivo* on both human subjects and rabbits showed that with high concentrations of the mixture of ascorbic acid and hydrogen peroxide there occurs a very pronounced spreading effect in the subcutaneous tissue and of the same magnitude as that produced with hyaluronidase. Since the oxidation of ascorbic acid was active only under aerobic conditions, it might possibly indicate that a labile peroxide was involved in the reaction. Barron *et al.*[84] suggested that hydrogen peroxide is probably formed during the autoxidation of ascorbic acid. It thus appeared possible that hydrogen peroxide may have been involved in producing the depolymerization effects even in those experiments in which it had not been added. Since the addition of pure catalase did not inhibit the reaction in such experiments, it seemed questionable[82] that peroxides were involved in the reaction. However, the catalase control of the situation has not been settled [85a, b, 86a, b] since peroxidases, pyruvates and compounds with free SH groups such as

[77] W. V. B. Robertson, M. W. Ropes, and W. Bauer, *Am. J. Physiol.* **126**, 609 (1939).
[78] W. V. B. Robertson, M. W. Ropes, and W. Bauer, *Biochem. J.* **35**, 903 (1941).
[79] D. McClean and C. W. Hale, *Nature* **145**, 866 (1940).
[80] G. Favilli, *Nature* **145**, 866 (1940).
[81] J. Madinaveitia and T. H. H. Quibell, *Biochem. J.* **35**, 453 (1941).
[82] B. Skanse and L. Sunblad, *Acta Physiol. Scand.* **6**, 37 (1943).
[83] W. Daubenmerkl, *Acta Pharmacol. Toxicol.* **7**, 153 (1951).
[84] E. S. G. Barron, R. H. DeMeio, and F. Klemperer, *J. Biol. Chem.* **112**, 625 (1935).
[85a] D. Cavallini, *Arch. fisiol.* **45**, 189 (1946).
[85b] E. Stotz, C. J. Harrer, M. O. Schultze, and C. G. King, *J. Biol. Chem.* **120**, 129 (1937).
[86a] M. G. Sevag, *Biochem. Z.* **267**, 211 (1933).
[86b] D. Cavallini, *Boll. soc. ital. biol. sper.* **22**, 169 (1946).

cysteine may also contribute appreciably to the decomposition of hydrogen peroxide.

(b) Chondroitin-Sulfuric Acid Complex. There is lack of accord among investigators as to variations in chondroitin-sulfuric acid in the mesenchymal supporting tissues of normal as compared to scorbutic animals. Höjer[7] found a collagen atrophy in scurvy especially marked in cartilage[87] in and near the columns of the proliferating zone, in and near the epiphyses. Sections stained with methylene blue did not appear to be different from normal in their presumable content of chondroitin-sulfuric acid. Meyer,[88] on the contrary, reported that cartilage sections of scorbutic guinea pigs were deficient in chondroitin-sulfuric acid. His observations were based on staining reactions. Sadhu[89] found that the chondroitin-sulfate content of skin and bones was considerably decreased in scorbutic animals. Bradfield and Kodicek[75] did not find marked difference in the toluidine blue-staining reactions of sections through skin wounds of scorbutic as compared to normal guinea pigs and interpreted the results as indicating that sulfated polysaccharides cannot account for the abnormal abundance of polysaccharide material which they found in the scorbutic wounds. Meyer and associates[29] have shown that the protein or proteins to which some of the sulfate esters of connective tissue are bound are distinct from collagen, since they contain tyrosine and tryptophan. Because of the importance of ascorbic acid for the metabolism of tryosine[90] it seems possible that the vitamin may perform a special function in those chondroitin-sulfuric acid complexes containing this amino acid.

Bradfield and Kodicek[75] evaluate the role of ascorbic acid with respect to the interfibrillar substance as follows: "Evidently vitamin C has a profound influence on mucopolysaccharide metabolism, but whether direct or indirect, and whether affecting the kinds of mucopolysaccharides produced, or the mucolytic enzymes for their disposal, is at present uncertain."

It is obvious from this discussion that many of the problems pertaining to connective tissue remain unsolved. The lack of specificity in the reactions of both the enzymes[91] and stains[57, 92-96] used in the histochemical studies

[87] The data for cartilage are presented since there are none for connective tissue.

[88] A. W. Meyer and L. M. McCormick, Studies in Scurvy, *Stanford Univ. Publ., Univ. Ser. Med. Sci.* **2** (1928).

[89] D. P. Sadhu, *Indian J. Physiol. and Allied Sci.* **6**, 49 (1952).

[90] R. R. Sealock, B. Ziegler, and R. L. Driver, *J. Biol. Chem.* **128**, lxxxix (1939).

[91] J. H. Humphrey, *Biochem. J.* **40**, 442 (1946).

[92] L. Lison, Histochemie animale, méthodes et problems. Gautier-Villars, Paris (1936).

[93] B. Sylvén, *Acta Chir. Scand.* **86**, *Suppl.* 66 (1941).

[94] K. Meyer, *Physiol. Revs.* **27**, 335 (1947).

[95] G. Wislocki, H. Bunting, and E. W. Dempsey, *Am. J. Anat.* **81**, 1 (1947).

[96] R. D. Lillie, *Anat. Record* **108**, 239 (1950).

results in serious limitations to their usefulness. Regardless of the difficulties and dangers involved in the use of these methods, however, we can doubtless look forward to substantial progress in this rapidly developing field of investigation.

In summary, it may be stated that present knowledge indicates definitely that ascorbic acid is essential for the production of fibers and for the maturation of reticulin to collagen. It also appears to have a role in the organization of the interfibrillar substance, possibly for the production of its protein components and/or their conjugation with the carbohydrate complex.

b. Bones and Cartilage

Just as in connective tissue, the effect of ascorbic acid on the bones and cartilage is chiefly in relation to collagen synthesis. Rogers *et al.*[97] found that collagen or collagen-like proteins comprised about 90 to 96 % of the organic matrix in the bones examined of rabbits, oxen, and humans. Lightfoot and Coolidge[41] found that the decrease from the normal values of collagen nitrogen of the bones in scurvy was marked in guinea pigs 10 days old, appreciable in those 36 days old, and much less in the older animals. Sadhu[89] reported that the chondroitin sulfate content of bones was considerably decreased in scurvy.

Lack of ascorbic acid produces a pathological condition at the growing ends of the bones characterized by a disorderly organization of the cells and a resulting weakness. The changes are to be found at the costochondral junctions and at the junction of the diaphysis and epiphysis. Aschoff and Koch[30] have studied the changes in bone structure in human scurvy, and Höjer,[7] Wolbach and Howe,[8] Meyer,[88] Ham and Elliott[98] and MacLean *et al.*[99] have investigated them in the guinea pig. Follis[100] describes and illustrates the changes in bone, and the reader is referred to his account for a more detailed picture than can be presented here.

In scurvy there is a failure of the osteoblasts to form osteoid tissue. Wolbach[101] describes their behavior as follows: "Formation of cartilage and bone matrices ceases, and the osteoblasts become elongated, assume the shapes of fibroblasts and migrate toward the diaphysis. Here these cells become surrounded by liquid, presumably a deficient product of continued activity toward matrix formation, and give rise to an apparent region of edematous connective tissue at the ends of the diaphysis, the Gerüstmark

[97] H. T. Rogers, S. M. Weidmann, and A. Parkinson, *Biochem. J.* **50**, 537 (1952).

[98] A. W. Ham and H. C. Elliott, *Am. J. Pathol.* **14**, 323 (1938).

[99] D. L. MacLean, M. Sheppard, and E. W. McHenry, *Brit. J. Exptl. Pathol.* **20**, 451 (1939).

[100] R. H. Follis, Jr., The Pathology of Nutrition Disease. Charles C Thomas, Springfield, Ill. (1948)

[101] S. B. Wolbach, *J. Am. Med. Assoc.* **108**, 7 (1937).

(framework marrow) of German authors." Eventually resorption of bone matrix with marked changes in the epiphyseal cartilage occurs. The cells of the periosteum cease to form bone, and the attachment of the trabeculae with the epiphyseal cartilage becomes weak, with a resulting tendency to fractures in the epiphysis and diaphysis. Fractures of the calcified matrix material result in a region of complete degeneration, the Trümmerfeld zone. Continued proliferation of osteoblasts of the periosteum causes the periosteum to become separated from the bone cortex. The osteoblasts increase in number, but no intercellular material (osteoid) is formed. Resorption of bone salts occurs, resulting in rarification of the bones, a change which Meyer[88] found to occur over the entire skeleton with all the large bones becoming smoother and appearing somewhat glassy. Although absorption in the bones was marked, he rarely found osteoclasts present. Harman *et al.*[102] found that guinea pigs after 4 weeks on an ascorbic acid-deficient diet showed resorption of the jaw bone at the bases of the molars and along the edge of the alveolar area. This was true for animals ranging in age from 20 days to 3 years. Robertson[67] found that significant losses of collagen from bones and teeth occur in scurvy. MacLean *et al.*[99] found that in acute deficiency of vitamin C in guinea pigs no characteristic histological changes occur in tissues other than in bones and teeth. It would thus seem probable that the changes in other tissues observed by various investigators were a consequence of a less acute deficiency of the vitamin, or possibly in some instances a deficiency of essential factors other than vitamin C. As to degenerative changes in the cartilage cells Meyer[88] observed that in vitamin C deficiency the cytoplasm may become vacuolated and rarefied and that later degenerative changes may occur in the nuclei and finally in the matrix resulting in complete loss of substance. Höjer[7] noted a collagen atrophy in the cartilage which was particularly marked in and near the columns of the proliferating zone. Some of the other workers have not found definite changes in cartilage, possibly because under their experimental conditions the scurvy progressed rapidly, allowing insufficient time for the changes to develop.

c. Teeth

Growth of the guinea pig's incisors is very rapid under conditions of good nutrition. A growth of 2 mm. per week occurs, in contrast to a few millimeters per year for humans.[103] Zilva and Wells[5] showed that pathological changes in the odontoblasts and dentine occur in scurvy. Höjer[7] made a very important contribution in which he showed that the growth and arrangement of the odontoblasts is directly related to the vitamin C intake.

[102] M. T. Harman, M. M. Kramer, and H. D. Kirgis, *J. Nutrition* **15**, 277 (1938).
[103] P. E. Boyle, *J. Dental Research* **14**, 172 (1934).

Other investigators[8, 98, 99, 104-106] have also added to our knowledge in this field. Follis[100] has concisely summarized the changes in the teeth in scurvy as follows:

"When guinea pigs are placed on a scorbutic diet alterations very soon appear in the odontoblasts; these cells become atrophic and soon resemble the nearby pulp cells. They lose their orderly polar arrangement and become completely disorganized. The vessels of the pulp become dilated and red cells ooze through. As a result of the changes in the odontoblasts, dentine is laid down irregularly and the dental tubules are arranged in haphazard fashion. Dentine deposition soon stops entirely. The predentine becomes hypercalcified. A few of the odontoblasts in the pulp apparently form some dentine, at least enough to enclose themselves. The alizarin technique has been employed to demonstrate that dentine formation is quantitatively related to ascorbic acid intake.[105] In the guinea pig changes in the enamel organ come later in the course of the deficiency. The ameloblasts atrophy and hemorrhages are encountered. Both these alterations have been interpreted to be due to traumatic injury of the enamel organ as a result of poor support. There is no evidence of any relationship between ascorbic acid deficiency and dental caries. There is rarification of the alveolar bone, as might be expected, when one recalls the changes encountered in the ribs and other bones of experimental animals and humans. Weakness of the supporting bones as well as weakness of the collagen fiber supporting apparatus allows for great mobility and decreased ability to withstand mechanical stresses encountered in chewing. The changes in the supporting structures of the guinea pig have been likened to the diffuse alveolar bone atrophy of pyorrhea encountered in the human.[106]"

2. Muscles

a. Voluntary

Ascorbic acid is of special importance in maintaining the integrity of both structure and function of muscle tissue, although it is present there only in low concentrations as compared to many other tissues. Both the sarcolemma and muscle tissue itself show degenerative changes in scurvy. Holst and Fröhlich[3] observed fatty degeneration of the muscles. Jackson and Moore[107] found that certain portions of muscle fibers are completely disintegrated. Aschoff and Koch,[30] however, did not find a fatty change of the fibers in their study of human scurvy but noted that often the fibers

[104] E. W. Fish and L. J. Harris, *Trans. Roy. Soc.* (*London*) **B223**, 489 (1933).
[105] P. E. Boyle, O. A. Bessey, and P. R. Howe, *Arch. Pathol.* **30**, 90 (1940).
[106] P. E. Boyle, O. A. Bessey, and S. B. Wolbach, *J. Am. Dental Assoc.* **24**, 1768 (1937).
[107] L. Jackson and J. J. Moore, *J. Infectious Diseases* **19**, 478 (1916).

located in hemorrhagic areas were shrunken and stained unusually deeply with eosin. Hart and Lessing[108] observed granules which stained like calcium in the muscles of monkeys dying of scurvy. Höjer[7] found that the muscles of scorbutic guinea pigs are affected by the disease at an early stage. In fact, this change was the first sign of scurvy. A wasting of the tissue occurred which was an atrophy combined with necrosis and a general hyperemia. He observed the appearance of certain giant nucleated cells and in some areas an impregnation of the necroses with calcium. Also, there were hemorrhages in the places exposed to mechanical strain or trauma. Meyer[88] found that hemorrhagic muscle may be changed so extensively in scurvy as to be scarcely recognizable in microscopic preparations. Marked hydropic degeneration was frequently observed, but fatty infiltration was found in only a few of his experimental animals. A pronounced coagulative change to a waxy state was observed in sections of hemorrhagic muscle. Usually in these areas, there was a great increase in the nuclei, the appearance of which and the absence of infiltration suggested that they probably arose from the sarcolemma. In some areas there was much, and in others little, evidence of lysis. Nothing but a foam-like residue remained in some places.

Dalldorf[109] observed the presence of ruptured cells in striated muscles in scurvy. Invariably there was degeneration of the intercostal muscles of scorbutic guinea pigs. He also noted that exercise would produce identical lesions in other skeletal muscles of scorbutic guinea pigs. The lesions thus appeared to be characteristic of the scorbutic state. Yakovlev[110] stated that experimental scurvy develops earlier and is fatal sooner in animals engaged in muscular work than in those at rest. He also found that muscular effort followed by a rest produced signs of scurvy more than does continuous work. Sekizima[111] observed multiplication, swelling, and atrophy of the sarcolemma, and in the muscle tissue itself he found swelling, atrophy, fragmentation, a tendency to bleeding, some fatty degeneration, slight calcification, and extensive waxy degeneration which he ascribed to an increase in hydrogen ion concentration. A disturbance in amino acid metabolism of scorbutic muscles[22] may possibly be involved in the observed histological defects. Boyle and Irving[112] found differences in the types of change in the muscles in acute and chronic scurvy in guinea pigs. In the acute form, hyaline degeneration of the muscles around the knee joint, ribs, scapulas, and tracheas was much less common and nuclear proliferation less pronounced

[108] C. Hart and O. Lessing, Der Skorbut der Kleinen Kinder. Ferdinand Enke, Stuttgart, 1913.
[109] G. Dalldorf, *J. Exptl. Med.* **50**, 293 (1929).
[110] N. N. Yakovlev, *J. Physiol.* (*U.S.S.R.*) **30**, 391 (1941).
[111] K. Sekizima, *Mitt. med. Akad. Kioto* **32**, 836 (1941).
[112] P. E. Boyle and J. T. Irving, *Science* **114**, 572 (1951).

than in chronic scurvy, but in the former the finer structure of the muscle became more prominent, the sarcolemma was reduced in amount, and the fibers showed more of a tendency to pull apart. Their results indicated that ascorbic acid is necessary for maintenance of the attachment of myofibrils to the sarcolemma. Hines *et al.*[113] studied the effect of different levels of ascorbic acid on neuromuscular regeneration in guinea pigs and found that regeneration in animals on suboptimal amounts of the vitamin was weaker than that of animals on optimal amounts. No special benefit resulted from excess amounts.

b. Involuntary

(1) *Heart.* Meyer[88] has been the chief contributor to our knowledge in this field. He found the scorbutic heart outwardly normal except that it was often flabby and pale, and he thought it likely that there may have been some hypertrophy of both ventricles. He found that the finer architecture of the heart muscle became obscured and the stain uneven in microscopic sections. Although a condition suggesting beginning hyaline degeneration occurs, he never observed the waxy degeneration which was sometimes so marked in the skeletal muscles.

(2) *Musculature of the Stomach, Intestine, Ducts, and Urinary Bladder.* Meyer[88] found that hemorrhages were usually less pronounced and common in the stomach than in the duodenum and cecum. The musculature became vacuolated and subject to lysis. Although edema may have been present, he considered it was not responsible for the vacuolated and fenestrated appearance of the musculature. As to the bladder he states that "when one considers the profound changes present in the mucosa and musculature, and probably also in the nerves of the bladder, one scarcely can wonder that urinary incontinence occurs in some of these animals."

The best available evidence at the present time indicates that swelling and atrophy of both the sarcolemma and of muscle tissue itself may be found. Hemorrhages occur frequently, and waxy degeneration may be observed in skeletal but not in heart muscle. There is some tendency to calcification, fragmentation, vacuolation, and lysis of cells.

3. Nervous System

The nervous system is considerably affected in scurvy. Meyer[88] found hemorrhages in brain, spinal cord, posterior root ganglia, and nerve trunks of scorbutic guinea pigs. He observed degenerative changes (lytic) other than fatty, resulting in complete loss of substance in the central, peripheral, and sympathetic nervous systems. He described the condition as follows:

[113] H. M. Hines, B. Lazere, J. D. Thompson, and C. H. Cretzmeyer, *J. Nutrition* **27**, 303 (1944).

"In earlier stages of this apparently waxy degeneration as revealed in cross sections, the fibers lose their circular outlines, become indistinct, and fuse more or less completely into larger masses. The axis cylinders are greatly swollen, the medullary sheaths are less evident, and the epineurium is separated from the contained fibers by a clear space which may result from edema." Holst and Frölich[3] had previously reported finding Wallerian degeneration sometimes associated with swelling and fragmentation of the axis cylinders. Meyer states that changes such as he observed could easily explain the occurrence of paralysis, although they do not account for the paralysis in the posterior extremities. He was of the opinion that it is failure of recovery from central lesions which is responsible for the permanent spastic condition observed in the posterior extremities of some animals. Sadhu[89] has recently reported finding that the brains of scorbutic guinea pigs contained significantly more cerebroside and less sphingomyelin than did the brains of normal animals.

4. Circulatory System

a. Blood Vessels

(1) *Capillaries.* There are numerous reports[114a, b, c, d] of increased capillary fragility in scurvy. Findlay[114e] followed the changes in the vascular system in guinea pigs maintained on a diet of oats and bran supplemented with autoclaved milk, and his observations led him to conclude that the chief lesion in scurvy is a swelling and degeneration of the capillary endothelium resulting in stagnation of the blood in the capillaries. Hemorrhages were considered as secondary to the breakdown of the intercellular substance. Hess[63] was also of the opinion that the chief defect was due to a lesion of the endothelial cells or their cement substance. This view has also been supported by Wolbach and Howe[8] and by Martin.[114f] Chambers and Cameron[114g] have stated, however, that ascorbic acid is not essential for the production and maintenance of the intercellular cement material in the epithelial tissues.

The evidence on which their conclusions were based was obtained in experiments with epithelial cultures of kidney and intestine from chick embryos. The effect of ascorbic acid on sheets of the growing tissues was studied. Cultures prepared with plasma and serum from scorbutic guinea

[114a] G. Dalldorf and H. Russell, *J. Am. Med. Assoc.* **104**, 1701 (1935).
[114b] L. J. Roberts, R. Blair, and M. Bailey, *J. Pediat.* **11**, 626 (1937); **15**, 25 (1939).
[114c] G. H. Bell, S. Lazarus, and H. N. Munro, *Lancet* **239**, 155 (1940).
[114d] J. Lavollay and J. Sevestre, *Compt. rend. soc. biol.* **220**, 472 (1945).
[114e] G. M. Findlay, *J. Pathol. Bacteriol.* **24**, 446 (1921).
[114f] W. C. Martin, *Western J. Surg. Obstet. Gynecol.* **50**, 508 (1942).
[114g] R. Chambers and G. Cameron, *Am. J. Physiol.* **139**, 21 (1943).

pigs ceased growing, but the cells did not separate and within a short time they deteriorated. In cultures in which the cells had become separated because of lack of calcium in the medium, it was stated that the presence of ascorbic acid was unnecessary for re-establishing the connections. From these results it was concluded that the intercellular cement does not require ascorbic acid either for its formation or for its maintenance. However, growth was definitely accelerated by the presence of the vitamin. The evidence for the above conclusions is not convincing. Since both embryo juice and plasma from normal animals were used in the preparation of the medium, it must have contained ascorbic acid. Also, since the medium formed a coagulum around the tissues, the washing for a short period probably did not remove all the ascorbic acid contained in it. Moreover, since the cultures were made from tissues of normal animals, they doubtless contained ascorbic acid within the cells and it would not have been removed by washing for a short period. Such traces of ascorbic acid remaining in the cells plus possible traces left in the surrounding medium may have been sufficient to permit restoration of the organic constituent of the cement when calcium was supplied but not enough to provide for any growth.

In view of these considerations the evidence that ascorbic acid is not necessary for the production or re-establishment of the intercellular cement is not conclusive. There is, in fact, considerable suggestive evidence from growth studies that there is a requirement for the production of intercellular cement though it is probably small as indicated by Galloway, Garry, and Hitchins'[56] finding of a low requirement of ascorbic acid for healing wounds of epithelial tissue. Presumably, the formation of the intercellular cement could be a factor in determining a possible need of the vitamin in epithelial tissues. It is obvious that challenging problems with respect to the intercellular cement of the capillary walls await solution.

Resuming the discussion on the effect of ascorbic acid on capillary resistance, mention should be made of a study by Zilva[115] on guinea pigs with an acute condition of scurvy in which he could detect no significant decline in the strength of the capillary walls. Höjer[7] attributed the hemorrhagic tendency in scurvy to weakness in the vascular walls and stated, in agreement with Iwabuchi,[116] that often the dilatation of the capillaries (and veins) was so great as to give an erroneous impression of actual hemorrhage. Although hemorrhages occur in vitamin C deficiency, no actual histological evidence of a change in capillary walls has been found.[117, 118] Extravasation may possibly occur, nevertheless, by the red cells oozing through

[115] S. S. Zilva, *Biochem. J.* **45**, 79 (1949).
[116] T. Iwabuchi, *Z. ges. exptl. med.* **30**, 65 (1922).
[117] S. B. Wolbach and O. A. Bessey, *Physiol. Revs.* **22**, 233 (1942).
[118] R. E. Lee and Z. N. Lee, *Am. J. Physiol.* **149**, 465 (1947).

the dilated walls.[100] Nungester and Ames[21] obtained results which suggested the possibility of capillary leakage. Intraperitoneal exudates obtained from scorbutic guinea pigs invariably contained large numbers of red blood cells whereas red cells were never found in exudates having a vitamin C content of over 0.45 mg. per 100 ml.

Another factor which may influence the strength of the capillaries is the integrity of the membrane surrounding them. Chambers and Zweifach[119] showed the presence of a mucopolysaccharide in the connective tissue sheath of the capillaries, and Penney and Balfour[57] pointed out that any failure in the formation of mucopolysaccharide might result in the weakening of the sheath, thus leading to hemorrhages. Gersh and Catchpole[76] have suggested that the strength of the capillaries is determined in part by the basement membrane surrounding them. They report finding evidence that depolymerization of this glycoprotein (membrane) occurs in scurvy and may lead to a weakening of the capillary wall. Further investigation of this aspect of the blood vessel problem will be awaited with interest.

The permeability of the capillaries of scorbutic and normal guinea pigs was studied by Elster and Schock.[120] The blue dye (T-1824) was administered intravenously, and no qualitative or quantitative differences could be observed in its distribution in the tissue except for extravasation into the periarticular soft tissues in the scorbutic but not in the control animals. Since they saw no evidence of capillary leakage, they concluded that capillary permeability was not altered by ascorbic acid deficiency in the guinea pig. However, as previously stated, results of Follis[100] and Nungester and Ames[21] suggest the possibility of capillary leakage. Chambers and Zweifach[119] are of the opinion that the intercellular cement of the capillary wall determines the permeability to diffusible substances whereas Landis[121] and Cowie *et al.*[122] hold that capillary permeability depends on the state of the entire endothelium. The latter investigators concluded from radioactive tracer studies that "diffusion rather than filtration is the predominant process in the exchange of substances across the capillary wall." Elster and Schock agree that it is possible that the intercellular cement could be affected in scurvy even though diffusion of the dye is still normal and their experiments do not definitely establish or exclude alterations in function of the intercellular substance. It seems quite possible to the writer that structural changes on a submicroscopic level, such as alterations in molecular orientation and/or destroying of linkages in basic constituents, particularly at cell surfaces, could be involved in a functional change caus-

[119] R. Chambers and B. W. Zweifach, *Physiol. Revs.* **27**, 436 (1947).
[120] S. K. Elster and J. A. Schock, *Am. J. Physiol.* **161**, 283 (1950).
[121] E. M. Landis, *Ann. N. Y. Acad. Sci.* **46**, 713 (1946).
[122] D. B. Cowie, L. B. Flexner, and W. S. Wilde, *Am. J. Physiol.* **158**, 231 (1949).

ing alteration in permeability. However, it is also possible that leakage of the capillaries is explainable by swelling of the walls without marked molecular disorientation.

(2) *Arteries and Veins.* Thin walls in both arteries and veins have been reported in scurvy;[7, 107] there is also some evidence of lytic processes resulting in disintegration of the vessel walls.[88] Hemorrhages occur frequently, but a hyperemic condition is often mistaken for a hemorrhage.[88] The partial disappearance of the collagen fibrils is considered to be an important factor in the pathogenesis of the hemorrhages.[7] In addition to this direct weakening of the vessel walls there is an effect caused by shrinking of the cells of the tissues which reduces the outer support for the atrophic vessel walls. Without this support the pressure from the inside presumably must have a still greater effect.[7]

(3) *Blood Pressure.* Selezeneva[123] found a great reduction in the response to intravenous injections of epinephrine in animals deficient in vitamin C. On the other hand, Lee and Holze,[124] using larger doses of the hormone, found no difference in blood pressure between the scorbutic and normal groups or in the ability to respond in pressor manner to injections of epinephrine. As compared to the normal control animals the scorbutic animals had a definitely lessened ability to withstand loss of blood.

b. Blood

The somewhat divergent reports of the blood picture in scurvy by different investigators are probably to be ascribed chiefly to differences in experimental conditions, such as nature of the diet, age of the animals, and degree of advancement of the scorbutic condition. Very young animals often fail to show definite blood changes because they die from scurvy too quickly for anemias to develop.[125] The extent of hemorrhage is probably of special significance in influencing the blood picture. Although knowledge of the dietary requirements of the guinea pig is far from complete, some of the earlier studies made on the blood could profitably be reinvestigated in the light of the present known facts. Undoubtedly some of the studies have been made with animals maintained on diets deficient in factors other than ascorbic acid.

(1) *Volume.* Harman and Kordisch[126, 127] observed an apparent change in blood peripheral volume during the progress of scurvy. In the early stages of the deficiency, blood could easily be obtained from any peripheral vessel

[123] A. A. Selezeneva, *Proc. Sci. Inst. Vitamin Research (U.S.S.R.)* **3**, 205 (1941).
[124] R. E. Lee and E. A. Holze, *Proc. Soc. Exptl. Biol. Med.* **76**, 325 (1951).
[125] H. C. S. Aron, *J. Nutrition* **18**, 375 (1939).
[126] M. T. Harman and M. S. Kordisch, *Trans. Kansas Acad. Sci.* **47**, 367 (1945).
[127] M. T. Harman, *Trans. Kansas Acad. Sci.* **53**, 319 (1950).

in the ear, but later it became very difficult and in some cases impossible to obtain it from the marginal vessels. Elster and Schock[120] reported noting no change in blood volume in scurvy. Further study is desirable, however, before drawing final conclusions as to the effects of scurvy on total blood volume.

(2) *Erythrocytes.* Decreases in erythrocytes have been reported by several investigators.[88, 114e, 125, 128, 129] McCormick[88] (Meyer and McCormick) made blood studies of the scorbutic guinea pigs of which Meyer[88] had described the gross and minute morphology. He found an average fall in erythrocytes of 30 % and a drop in hemoglobin values of 38 %. Since smears of the bone marrow showed the presence of an increased number of nucleated red cells as compared to normals, it seemed that there was an increased blood destruction and stimulation of the bone marrow. Smears from the bone marrow did not show reticulated cells but did show many nucleated erythrocytes. Blood smears, however, revealed large vacuolated reticulated cells. McCormick concluded that "whatever the cause of the red cell destruction the young cells are more susceptible to its influence." Hemorrhages seemed to be the most likely cause of the destruction of red cells.

Mettier and Chew[130] studied blood formation in relation to ascorbic acid nutrition in guinea pigs with an initial weight of 300 to 500 g. The survival time on the deficient diet usually ranged from 21 to 30 days. Coincident with the first appearance of gross symptoms of scurvy, which occurred usually from the tenth to the fifteenth day, an anemia developed. The red cells decreased from an average normal of about 5,000,000 per milliliter to half that number. With the administration of 3 ml. of orange juice daily, there was prompt improvement in the blood picture. An increase in reticulocytes in the peripheral blood was observed and an increase in mitotic figures in the bone marrow. Azodi[131] conducted tests with guinea pigs fed a diet of sterilized milk and zweiback with a supplement of cod liver oil. This diet was possibly inadequate with respect to copper and iron, some of the B vitamins, and vitamin E. During the first week of deprivation of ascorbic acid he observed a 20 % increase in the number of red cells. The quite young animals usually showed definite evidence of scurvy by the second to third week. By this time the red cells and hemoglobin showed a decrease. In animals which lived beyond this time there was a marked drop in erythrocytes and hemoglobin. In young as well as in full-grown animals the increase in erythrocytes was greater than that of hemoglobin during the early phases of scurvy so that the color index fell. Later, as the number

[128] D. Liotta, *Arch. farmacol. sper.* (*Rome*) **36,** 76 (1923).
[129] K. I. Kondo, *Folia Pharmacol. Japon.* **32,** 192 (1941).
[130] S. R. Mettier and H. B. Chew, *Proc. Soc. Exptl. Biol. Med.* **29,** 11 (1934).
[131] Z. Azodi, *Biochem. Z.* **291,** 34 (1937).

of red cells decreased, their fall was greater than that of hemoglobin and the color index approached that of normal values. May *et al.*[132] produced megaloblastic anemia in monkeys on a diet deficient in ascorbic acid.

(3) *Leucocytes.* Variable results have been reported by different investigators as to the effect of ascorbic acid on leucocyte counts. Harman and Kordisch[126] reported a tendency for a decreased leucocyte count in ascorbic acid-deficient animals. However, the difference as compared to normal animals was both small and inconstant. McCormick[88] obtained increases in leucocytes in 92% of his animals with an average increase equal to 80%; the polymorphonuclear cells were increased in 98% of the cases with an average increase in number equal to 233%; the lymphocytes decreased in 98% of the cases with an average decrease equal to 64%. The results suggested very distinct changes in the blood picture except with respect to monocytes, basophiles, eosinophiles, and transitionals. Since the leucocytosis ended quickly upon the resumption of antiscorbutics, he thought it unlikely that it was due to infection. In fact, gross signs of infection were not found in any of his experimental animals. However, increased leucocytosis during infection may cause an increased utilization of the vitamin;[133] increased metabolism may possibly also cause an increased usage.[54] Azodi[131] found that an increase in leucocytes occurred at the time the red cells began to decrease. The white cells increased 89% whereas the red cell decrease was 34%. In the qualitative white cell picture there was an increase in segmented cells and a decrease in lymphocytes. Lymphocytosis was changed to neutrophilia during the very early phases of the deficiency, but in the third week this relation was reversed. The number of lymphocytes began to increase and became even greater than the normal. In young animals the increase in lymphocytes was concomitant with the onset of anemia. The influence of ascorbic acid was to further increase the red cells and decrease the white. Other investigators[134-136] have also shown a leucopenic effect of ascorbic acid.

The increase in number of leucocytes frequently found in scurvy may be either a consequence of increased cell production or more probably of decreased phagocytic activity[21] and lessened migration from the blood stream to the tissues. A decrease in adrenal cortical hormones would presumably produce the latter effect. The concentration of the vitamin in the blood falls continuously during the course of the deficiency until, if the animal continues to live, the zero point is reached. The plasma and red cells are

[132] C. D. May, R. D. Sundberg, and F. Schaar, *J. Lab. Clin. Med.* **36**, 963 (1950).
[133] T. D. Cuttle, *Quart. J. Med.* **7**, 575 (1938).
[134] A. Böger and W. Martin, *Münch. med. Wochnschr.* **82**, 899 (1935).
[135] H. J. Spörl and R. Pongratz, *Klin. Wochnschr.* **15**, 1639 (1936).
[136] I. S. Wright and A. Lilienfeld, *Arch. Internal Med.* **57**, 241 (1936).

depleted rather quickly, but a considerable amount remains in the white cells until the signs of scurvy appear.[137-139] In advanced scurvy this retention by the white cells is presumably, in part, a consequence of their original high content as shown by Stephens and Hawley[16] and later confirmed by Butler and Cushman.[138, 139] It also appears that the leucocytes must have a special capacity to absorb and retain the vitamin.

(4) *Platelets.* Hess and Fish[140] reported that the number of blood platelets was normal in infantile scurvy. Tobler[141] and Brandt[142] observed an increase in the platelets in human scurvy. Hess[63] confirmed this observation and stated that it was one which had not been anticipated in connection with a disorder characterized by hemorrhage. However, several investigators[129, 143, 144] found a thrombocytopenia associated with scurvy. Little is known of the factors affecting blood platelets in the guinea pig. The need of further study in this field is obvious.

c. Coagulation Mechanism

Hess and Fish[140] observed a slight diminution of the clotting power of the blood in human scurvy which was not a result of insufficient calcium in the diet. Presnall[144] found that a 54 % delay in clotting time accompanied the onset of scurvy in guinea pigs. Kühnau and Morgenstern[145] noted that ascorbic acid had a marked accelerating effect on the *in vitro* coagulation of the blood in the presence of minimal quantities of heavy metals. Hanut[146] found that the intravenous injection of ascorbic acid (10 to 38 mg. per kilogram) decreased the coagulation time of recalcified oxalated plasma from 9 to 25 % but similar injections into guinea pigs beginning to show signs of scurvy had little or no effect. Marx and Bayerle[147] stated that the coagulation time, determined on oxalated plasma, is normal in scurvy. The general trend of evidence thus indicates that there is either no change or a slight delay in clotting during the onset of scurvy.

Since scurvy is a hemorrhagic disease, consideration should be given to the factors concerned in the coagulation mechanism. The primary factors involved are (1) prothrombin, (2) thromboplastin, (3) ionized calcium,

[137] M. Heinemann and P. M. Hald, *J. Clin. Invest.* **19**, 469 (1940).
[138] A. M. Butler and M. Cushman, *J. Biol. Chem.* **139**, 219 (1941).
[139] A. M. Butler and M. Cushman, *J. Clin. Invest.* **19**, 459 (1940).
[140] A. F. Hess and M. Fish, *Am. J. Diseases Children* **8**, 385 (1914).
[141] W. Tobler, *Z. Kinderheilk.* **18**, 63 (1918).
[142] H. Brandt, *Arch. Kinderheilk.* **17**, 395 (1919).
[143] S. Wasserman, *Folia Haematol.* **23**, Part 1, 1 (1918).
[144] A. K. Presnall, *J. Nutrition* **8**, 69 (1934).
[145] J. Kühnau and V. Morgenstern, *Hoppe-Seyler's Z. physiol. Chem.* **227**, 145 (1934).
[146] C. J. Hanut, *Compt. rend. soc. biol.* **121**, 1338, 1341 (1936).
[147] R. Marx and H. Bayerle, *Biochem. Z.* **315**, 366 (1943).

(4) fibrinogen, and (5) antithrombin. The following effects of scurvy with respect to these primary factors have been observed:

(1) *Prothrombin.* Sullivan *et al.*[148] reported that total and partial depletion of vitamin C in the guinea pig resulted in no change in the prothrombin level or activity of plasma diluted to one-eighth its original concentration. However, the clotting time of whole plasma was increased slightly. Marx and Bayerle[147] found, on the contrary, that in severe scurvy the prothrombin time definitely is prolonged and that a sufficient supply of ascorbic acid will bring it back to normal.

(2) *Thromboplastin.* The effect of scurvy on blood platelets has been reviewed in the section on blood. There are both qualitative and quantitative problems with respect to blood platelets remaining to be solved. They are of unquestionable importance in view of possible relations of thrombocyte disturbances to vascular clotting.

(3) *Calcium.* Hess and Killian[149] obtained contradictory results in regard to the calcium content of the blood in human scurvy. Their earlier studies had shown a decrease in calcium but later they found normal values. Humphreys and Zilva[150] in studies with guinea pigs observed that the blood calcium remains normal at least until the final stages of the disease. Randoin and Michaux[151] obtained similar results. This apparent constancy of the blood calcium level during a period of profoundly disturbed metabolism is doubtless in part possible because of withdrawals of calcium from the bony trabeculae.[152]

(4) *Fibrinogen.* Randoin and Michaux[151] and Marx and Bayerle[147] found an increased fibrinogen concentration in scurvy. Sullivan *et al.*[153] also observed that the fibrinogen content of guinea pig plasma increases markedly with the onset of scurvy. Within two weeks after administration of vitamin C the levels returned to normal. Salmon and May[154] found that plasma fibrinogen is considerably elevated in scurvy in the monkey. Other factors such as hemorrhages and infection may contribute to the increase but do not appear to account for it entirely.

(5) *Antithrombin.* Quick[155] holds that heparin does not by itself act as an antithrombin but merely intensifies the action of normal antithrombin of blood, i.e., serum albumin. If this is so, the diminution of plasma proteins[156]

[148] W. R. Sullivan, E. O. Gangstad, and K. P. Link, *J. Biol. Chem.* **151**, 477 (1943).
[149] A. F. Hess and J. A. Killian, *Proc. Soc. Exptl. Biol. Med.* **16**, 43 (1918).
[150] F. E. Humphreys and S. S. Zilva, *Biochem. J.* **25**, 579 (1931).
[151] L. Randoin and A. Michaux, *Compt. rend. soc. biol.* **194**, 565 (1932).
[152] J. D. Robertson, *J. Roy. Soc. Arts* **91**, 358 (1943).
[153] W. R. Sullivan, E. O. Gangstad, and K. P. Link, *J. Biol. Chem.* **152**, 367 (1944).
[154] R. J. Salmon and C. D. May, *J. Nutrition* **46**, 515 (1952).
[155] A. J. Quick, *Am. J. Clin. Pathol.* **19**, 1016 (1949).
[156] J. Doi, *J. Oriental Med.* **28**, 209 (1938).

and changes in the albumin/globulin ratio become of special importance in connection with the blood clotting problem. There is, in fact, considerable evidence that the albumin/globulin ratio becomes depressed in scurvy. Böger and Schroeder[157] studied the blood proteins in scurvy, then injected ascorbic acid and 3 to 4 days later again determined the proteins. An appreciable increase occurred in the albumin fraction. Ciatti and Auerbach[158] found an increase in blood proteins in guinea pigs with scurvy but the increase was confined to the globulin fraction. The albumin decreased, causing an appreciable change in the ratio. Garta[159] found a depression in the albumin/globulin ratio but no characteristic changes in total protein. Administration of large doses of ascorbic acid caused a rise in the ratio and also a rise in the total protein. Salmon and May[154] reported that total plasma protein did not appear to be altered in scurvy in monkeys.

Linneweh and Gen[160] studied hypoproteinemia and hypocalcemia in dogs and found that the calcium level depends upon the albumin ratio in the blood. It would be of interest to know if this relation between calcium and albumin also exists in guinea pig blood. If such were found, a functional relation between calcium and ascorbic acid would possibly be indicated.

d. Summary of the Effects of Scurvy on the Circulatory System

Lack of ascorbic acid produces disturbances in the circulatory system. The vessels, the blood, and, to some extent, the clotting mechanism are all affected. Histological changes in the capillaries have not been observed although alterations affecting permeability as a result of swelling and congestion may possibly occur. The effect of lack of the vitamin on the intercellular cement of the capillaries has not been definitely determined. The hemorrhagic areas seen in scurvy may result from a weakening of the connective tissue sheath surrounding the blood vessels. Definite changes in blood pressure have not been found. Changes in the blood itself include a decrease in volume, hemoglobin, number of red cells, and lymphocytes, with a tendency to increase in number and decrease in activity of the polymorphonuclear cells. The clotting time is either not changed or is slightly increased in scurvy, and the fibrinogen is definitely increased. The blood level of calcium does not change although definite changes occur in the calcium metabolism of the tissues. There tends to be a diminution of plasma proteins and a reduction in the albumin/globulin ratio.

[157] A. Böger and H. Schröder, *Klin. Wochschr.* **13,** 842 (1934).
[158] P. Ciatti and R. Auerbach, *Riv. clin. pediat.* **34,** 385 (1936).
[159] I. Garta, *Biochem. Z.* **290,** 364 (1937).
[160] F. Linneweh, F. Gen, and S. Gen, *Monatschr. Kinderheilk.* **83,** 337 (1940).

5. Other Organs

a. Liver

The most notable gross symptoms of ascorbic acid deficiency in the liver are paleness and a tendency to fatty infiltration. Congestion is common, and sometimes hemorrhages occur although there is no correlation between the degree of hepatic change and the severity and frequency of subcutaneous hemorrhages. In addition to the fatty infiltration there may be degenerative lytic changes and necroses which often show calcifications.[7, 161] Höjer[7] observed that the cells became reduced in volume, the cytoplasm was stained only faintly and appeared to be homogenized, and the cell boundaries were indistinct, with a tendency for diminution of parenchyma cells and an increase of stroma cells. Meyer[88] observed that disintegration of the hepatic parenchyma occurs and "as the cell walls disappear, a number of cells may coalesce, forming a single large clear area which may be surrounded by parenchyma cells of about normal size."

Murakami[162] obtained experimental evidence that lack of the vitamin causes a decrease in the amount of the bile and in the pigment-secreting function of the liver. Injection of the vitamin resulted in a restoration of these functions.

Russell and Callaway[163] found that trypan blue injected subcutaneously is more heavily deposited in the liver parenchyma cells of scorbutic than of normal guinea pigs. A fatty metamorphosis of the liver cells was also observed. This alteration together with the pathological deposition of the dye was considered as evidence of hepatic damage and as affording an explanation of the altered amino acid metabolism characteristic of ascorbic acid deficiency.[22, 90]

b. Pancreas

Hess[63] reported that the pancreas is normal in scurvy, and, since Höjer[7] did not mention the pancreas in the report of his studies on scurvy, he apparently found no definite abnormalities. The most noticeable changes which Meyer[88] found in some of the glands were the presence of vacuolation in the parenchyma cells, the occurrence of fatty infiltration throughout the organ, and desquamation of the epithelium of the ducts. Löwy[164] stated that the pancreatic islands are larger than normal and are hypoplastic.

[161] O. A. Bessey, M. L. Menten, and C. G. King, *Proc. Soc. Exptl. Biol. Med.* **31**, 455 (1934).

[162] O. Murakami, *Jap. J. Gastroenterol.* **11**, 1 (1939).

[163] W. O. Russell and C. P. Callaway, *Arch. Pathol.* **35**, 546 (1943).

[164] E. Löwy, *Z. ges. exptl. Med.* **38**, 407 (1923).

Banerjee[165] also observed that the number and size of the islets of Langerhans are much increased in scorbutic guinea pigs. The beta cells were degranulated in the scorbutic animals, but no degenerative changes in the cytoplasm were observed. Kristal[166] in a cytological study of the pancreatic tissue in ascorbic acid deficiency in guinea pigs observed no changes in the islet tissues.

c. Adrenals

Rodoni[167] was the first to report that the adrenals frequently are enlarged in scurvy, a finding which has been confirmed by several other investigators.[168-176] Hess[63] stated that hemorrhages mainly in the medullary region are commonly seen in scorbutic guinea pig adrenals, enlarging them greatly and giving them a deep red color. Hart and Lessing[108] found calcium deposits in the adrenals of scorbutic monkeys. Findlay[114e] and Iwabuchi[116] observed congestion in both the cortex and the medulla of scorbutic guinea pig adrenals. Höjer[7] stated that scorbutic guinea pigs "show early changes in the adrenals but not earlier than in other parenchymatous organs. These changes must, therefore, by no means be taken as a reason for placing the adrenals in the center of the pathology of scurvy." In a study of over 100 animals Höjer observed hemorrhages in the adrenals only once. He found the glands were somewhat enlarged in scurvy. Meyer[88] found the adrenals congested, usually slightly enlarged but not grossly hemorrhagic. He is of the opinion that it is misleading to say that there is true hypertrophy of the adrenals in scurvy. None of his scorbutic animals showed either hypertrophy or hyperplasia. The increase in size he found was due to congestion and fatty infiltration. Microscopic studies showed that the most notable increase of fat was to be seen in the fasicular and reticular zones and very little in the glomerulosa. The cell outlines often were not discernible. Gergely[177] reported that the cells of scorbutic glands showed a greater increase in

[165] S. Banerjee, *Ann. Biochem. Exptl. Med.* (*India*) **4**, 37 (1944).
[166] J. Kristal, *S. African J. Sci.* **41**, 273 (1945).
[167] P. Rondoni, *Brit. Med. J.* **I**, 542 (1919).
[168] V. K. LaMer and H. L. Campbell, *Proc. Soc. Exptl. Biol. Med.* **18**, 32 (1920).
[169] Y. Morikawa, *Endocrinology* **4**, 615 (1920).
[170] E. F. Robb, G. Medes, J. F. McClendon, M. Graham, and I. J. Murphy, *J. Dental Research* **3**, 39 (1921).
[171] R. I. McCarrison, *Indian J. Med. Research* **7**, 188 (1919).
[172] D. H. Bessessen, *Am. J. Physiol.* **63**, 245 (1923).
[173] A. J. Quick, *Proc. Soc. Exptl. Biol. Med.* **39**, 753 (1933).
[174] S. A. D'Angelo, A. S. Gordon, and H. A. Charipper, *Anat. Record* **81**, *Suppl.* 184, 185 (1941).
[176] S. Banerjee, *Ann. Biochem. Exptl. Med.* (*India*) **4**, 67 (1944).
[176] E. Nadel, E. S. Josephson, and A. S. Mulay, *Endocrinology* **46**, 253 (1950).
[177] J. Gergely, *Vitamine u. Hormone* **4**, 367 (1943).

volume than did those of normal adrenals and concluded that enlargement of the glands in scurvy appeared to be a result of simple swelling rather than a true hypertrophy. Stepto *et al.*,[178] however, held that there is a true hypertrophy, since there in an actual increase in dry weight, particularly in the protein content.

Bessey *et al.*[161] reported that the most characteristic lesion in the adrenals of scorbutic guinea pigs was the depletion of fat and cholesterol from the cortex in the final stages of the disease with little reduction in the fat content until several days before death. Their results confirmed Nagayama and Tagaya's[179] previous finding of a decrease of cholesterol in scurvy. The amount of cholesterol appeared to be directly proportional to the amount of fat in the fasiculata. That the decrease in adrenal lipids may be a consequence of inanition was suggested by later studies of Baldwin *et al.*[180] in which they found little difference in the amounts of lipids between scurvy animals and pair-fed controls. Blumenthal and Loeb[181] showed that enlargement of the adrenals in the guinea pig may occur as a result of underfeeding. However, results obtained by Oesterling and Long[182a] indicated an effect of lack of the vitamin on adrenal lipids not attributable to inanition. In paired-feeding experiments with animals somewhat older than those employed in the experiments of Baldwin *et al.*, they found that in early scurvy (17 days after the last vitamin C injection) the adrenal cholesterol was significantly higher than that of the controls, whereas in late scurvy (27 days after the last injection) there was a marked decrease in cholesterol compared with that of the pair-fed controls. Stepto *et al.*,[178] on the other hand, reported a progressive decrease in cholesterol concentration in the adrenals beginning between the seventh and fourteenth day of depletion.

Hoerr[182b] observed that in acute scurvy in guinea pigs the cells of the adrenals did not show marked degenerative changes but in the chronic form there was some degeneration of the reticularis and hypertrophy of the endothelium. Hemorrhages, if present, were small. The most notable change in the cells was in their increased lipoid content. All cells from the glomerulosa to reticulosa appeared fatty.

Fox and Levy[183] described a flabby, wrinkled condition of the adrenals of guinea pigs which had been supplied with partially oxidized orange juice as an antiscorbutic. On microscopic examination, the medulla ap-

[178] R. C. Stepto, C. L. Pirani, C. F. Consolazio, and J. H. Bell, *Endocrinology* **49**, 755 (1951).

[179] T. Nagayama and T. Tagaya, *J. Biochem.* (*Tokyo*) **11**, 225 (1929).

[180] A. R. Baldwin, H. E. Longenecker, and C. G. King, *Arch. Biochem.* **5**, 137 (1944).

[181] H. T. Blumenthal and L. Loeb, *Am. J. Pathol.* **18**, 615 (1942).

[182a] M. J. Oesterling and C. N. H. Long, *Science* **113**, 241 (1951).

[182b] N. Hoerr, *Am. J. Anat.* **48**, 139 (1931).

[183] F. W. Fox and L. F. Levy, *Biochem. J.* **30**, 211 (1936).

peared normal but the cortical cells showed fatty changes. Some of them had foamy contents; others were distended with large drops of fat. Attempts to repeat these findings of effects presumably due to dehydroascorbic acid and other breakdown products of ascorbic acid have not been reported.

The conflicting reports on the effect of vitamin C on the structure and content of the adrenal glands lead to the conclusion that further study of the problem is necessary.

d. Spleen

The spleen becomes somewhat enlarged and congested in scurvy. Höjer[7] sums up the changes which he observed as "an atrophy of the lymphoid tissue. To this has to be added hemosiderosis and sometimes hemorrhages." Meyer[88] found that the spleens of scorbutic animals tended to be somewhat congested and sometimes enlarged and firmer than those of normal animals. Microscopic examination showed that degenerative changes were usually to be found.

e. Gastrointestinal Tract

Bleeding, spongy gums are sometimes found in human scurvy. Ralli and Sherry[184a] state that "the gum change that does seem to be due to a deficiency of vitamin C is the hemorrhagic lesion which is associated with an accumulation of blood in the gums. Many of the other gum changes are probably due to underlying dental pathology." In contrast to human scurvy the condition in the guinea pig is not characterized by hemorrhages or ulceration of the gums.

The salivary glands undergo an atrophy in scurvy which Höjer[7] states "can be demonstrated at an early stage, somewhat later than the first changes in the teeth but earlier than the costal rib bone changes."

Hess[63] states that "lesions of the stomach are neither characteristic nor as a rule very striking. Hemorrhages occur, the larger ones generally in the subperitoneal layers, the smaller ones in any of the coats. Thickening of the wall follows or accompanies these hemorrhages. Superficial erosions of the mucosa or even ulcers may be seen." Since Höjer did not mention stomach pathology, apparently he observed no characteristic changes in scurvy. Meyer[88] found that ulceration of the mucosa was common in guinea pig scurvy but he did not find fatty degeneration of the mucosa. Congestion and hemorrhages were not unusual. Considerable evidence is accumulating that low food intake may be an important factor in the development of hemorrhages of the gastrointestinal tract.

[184a] E. P. Ralli and S. Sherry, *Medicine* **20**, 251 (1941).

Hess[63] stated that the intestine may show a variety of lesions in scorbutic human subjects which may progress to necrosis and extensive ulceration. Meyer[88] found congestion of and hemorrhages into the walls of the duodenum very common in the guinea pig but ulceration was not often observed. He found that the changes in the duodenum, ileum, cecum, and colon were very similar. Smith[184b] found "no significant impairment of the gastrointestinal tract of the guinea pig in either acute or chronic scurvy." If, however, the deficiency is imposed on the animal during the period of embryonic development, striking effects may be seen. In sections of the intestine of normal 27-day embryos Harman and Warren[38] found that "the epithelial lining was composed of tall columnar cells with distinct cell walls and well-defined nuclei. A definite basement layer was present. Also there was a distinct lumen in the intestine. The section of the intestine of the embryo of the experimental animal which had been on the deficient diet only 6 days showed no definite cell boundaries, the nuclei seemed to be run together, and the whole epithelial lining was sloughed off."

f. Genitourinary Organs

Kramer *et al.*[185] found that the ovaries of animals which had been on a diet devoid of vitamin C for 2 weeks or more showed signs of degeneration. Saffry *et al.*[186] observed that guinea pigs fed a vitamin C-free diet showed a severe condition of endometrial hyperplasia in contrast to the normal uterine tissue of the controls. Lindsay and Medes[187] showed that atrophy and degeneration of the germinal epthelium in scurvy destroys the reproductive function of the male at an early stage of the deficiency.

The kidneys are usually paler and often somewhat enlarged in scurvy. Hemorrhages may be found especially in the bladder, both in man and in the guinea pig.[63] Höjer[7] found changes occurring in the kidney which may be summarized as "an atrophy often combined with necrosis and secondary calcification." He found the epithelium of the kidney to be somewhat less sensitive to the deficiency than the cells of the liver and the spleen. Calcium deposits were sometimes present which he was inclined to interpret as indicating a strong calcium excretion through the kidney. However, no analyses were made of the calcium content of the urine. Meyer[88] observed that hemorrhages both focal and diffuse were common in all portions of the kidney and destruction of the renal epithelium was complete in places. The kidneys of Russell and Callaway's scorbutic guinea pigs[163] were more

[184b] E. A. Smith, *Am. J. Physiol.* **80**, 288 (1927).
[185] M. M. Kramer, M. T. Harman, and A. K. Brill, *Am. J. Physiol.* **106**, 611 (1933).
[186] D. Saffry, M. T. Harman, and M. M. Kramer, *Trans. Kansas Acad. Sci.* **40**, 389 (1937).
[187] B. Lindsay and G. Medes, *Am. J. Anat.* **37**, 213 (1926).

deeply stained than those of the normal controls when trypan blue was injected, suggesting a pathological change in the parenchyma.

g. Respiratory Organs

There are a few reports of extensive study of the lungs in scurvy. Meyer[88] found that the lungs of many of his guinea pigs showed "little or no change but very commonly small areas were dark red and sometimes this was true of an entire lobe or lung even, suggesting the existence of a broncho or lobar pneumonia. Microscopic examination, however, never confirmed this" (pneumonia). Congestion, collapse, and autolysis were sometimes observed. Microscopic studies showed autolytic changes in which the walls of some of the arteries had undergone almost complete lysis and cartilage cells in various stages of lysis were common. The most striking change was the presence of abundant vacuolation. Suitable stains revealed evidence of fatty degeneration.

h. Skin

The water content of the skin increases in scurvy. Hartzell and Stone[50] found that the skin of the abdominal wall of normal guinea pigs contained 76.1% moisture whereas that of scorbutic animals contained 79.1%. Bourne[188] found that large doses of ascorbic acid administered to rats may cause a loss of water from the skin even in normal animals.

The usual scorbutic skin lesions are petechiae occurring near the hair follicles. Wiltshire[189] reported that in a large proportion of his cases of human scurvy follicular keratosis was the first symptom of scurvy. It has been thought possible that other deficiencies such as lack of vitamin A may have been involved in producing the lesions, but Crandon, Lund, and Dill's[190] production of the condition in experimentally induced human scurvy affords convincing evidence that the condition can be caused by lack of ascorbic acid alone. A comparable condition has not been described in the guinea pig, although Sadhu[89] reported finding a considerable decrease in the chondroitin sulfate content in the skin of scorbutic animals. Harman and Warren[38] found a marked contrast in the structure of the skin of 27-day embryos of normal and vitamin C-deficient animals. Skin of the control embryos showed "a well-defined thickened epidermal layer of cells. The underlying cells had sharply defined cell walls and large, distinct well-defined nuclei. In the sections of the skin of the embryo of the experimental mother which had been on the deficient diet only 6 days the outer layer

[188] G. H. Bourne, *Lancet* **246**, 688 (1944).
[189] H. Wiltshire, *Lancet* **II**, 564 (1919).
[190] J. H. Crandon, C. C. Lund, and D. B. Dill, *New Engl. J. Med.* **223**, 353 (1940).

was not thickened and the cell walls of the underlying cells were indistinct and rather blurred in appearance. The nuclei were small and not well-defined."

i. Other Endocrine Glands

Some investigators find little evidence of an effect of ascorbic acid deficiency on the size or activity of the thyroid.[191, 192] Aschoff and Koch[30] stated that the thyroids and hypophyses were normal in their scurvy cases. Harris and Smith,[193] however, obtained results indicating a pathological condition of the thyroid in vitamin C deficiency in guinea pigs. The changes noted in the chronic deficiency were an increased number of interfollicular cells, a decrease in colloid, an increased vacuolization, and a lengthening of the cells lining the follicles. In acute scurvy, the changes were similar though less marked because of the shorter period of survival. Acute starvation by their methods revealed no changes in the thyroid. In similar studies, however, Murray[194] reported that in scurvy the thyroid shows hyperemia, hypersecretion, and irregularity of structure, and Abercrombie[195] described changes similar to those obtained by Harris and Smith. Meyer[88] stated that sections from some of the thyroids of his scorbutic animals "suggested a reduction in the amount of colloid and an increase in the amount of intrafollicular (desquamated) cells, but since both these intrafollicular constituents varied considerably in amount in the same thyroid, as well as in different thyroids, one needs a very large series in order to make reliable conclusions." It is obvious that the effects of ascorbic acid deficiency on the thyroid are not well differentiated and defined. In their study of the effects of ascorbic acid deficiency on embryonic development Harman and Warren[38] found that the thyroids in the normal fetuses were large, pale in color, and sharply convoluted whereas those of the deficient animals were smaller, darker in color, and only slightly convoluted.

6. Some General Cytological Aspects of Vitamin C Deficiency

A reduction in cytoplasm together with indistinctness of cell walls have been described by several investigators.[7, 38, 88] The increase in nuclei which has been found in several types of tissue is presumably a result of the disappearance of the normal cell boundaries, including the intercellular cement. These changes are undoubtedly of great importance.

[191] A. W. Spence and E. F. Scowen, *Biochem. J.* **29**, 562 (1935).
[192] M. Schober, *Endokrinologie* **22**, 24 (1939).
[193] K. D. Harris and A. E. Smith, *Am. J. Physiol.* **84**, 599 (1928).
[194] M. Murray, *Z. Vitaminforsch.* **6**, 239 (1937).
[195] W. F. Abercrombie, *J. Am. Med. Assoc.* **105**, 310 (1935).

D. FUNCTIONAL ASPECTS

1. Functions, Locus of Action, Mode of Action

a. Functions Attributed to Ascorbic Acid

1. It regulates the colloidal condition of intercellular substances having collagen or related substances as basic constituents. This is its most definitely established function.

2. It protects hydrogen carriers, thereby preventing injurious oxidations by acting as an antioxidant. An example of this type of action is to be found in its relations with α-tocopherol.

3. It promotes certain types of oxidation such as that of fatty acids and the production of the oxy type of adrenal cortical hormones.[196]

4. It preserves a proper balance between different enzyme systems; the oxidation-reduction potentials are thus maintained at certain levels.

5. It plays a role in the formation of desoxyribonucleic acid, a substance held to control the physical-chemical properties of the nuclei.[197]

b. Locus of Actions

(1) *Directly on the Protoplasm.* This occurs particularly in the special mesenchymal cells—fibroblasts, osteoblasts, odontoblasts, ameloblasts, etc. Without ascorbic acid there is a failure in these special cells to differentiate, mature, migrate, assume a proper orientation, and consequently a general inability to function properly. Adherents of the view of direct protoplasmic action have been Hansen,[198] Mall,[199] Lewis,[200] Höjer,[7] Meyer,[88] Fish and Harris,[104] Ham and Elliott,[98] and MacLean *et al.*[99] Höjer,[7] whose studies and their presentation did much to develop this concept, summarizes his observations as follows: "Scurvy reduces the nutritional state and the active function of probably all the most active, most rapidly growing and highest differentiated cells of the body. This reduction begins with the absence of antiscorbutic in the food. It becomes manifest at a rate that varies for the different cells and tissues: first in the odontoblasts, then in the osteoblasts, muscle cells, lymphoid tissue, fibroblasts and salivary glands; then in the liver, adrenals, chondroblasts, and last in the epithelium of the kidney. The order in which the changes set in and continue in odontoblasts, osteoblasts and chondroblasts seem to argue for the notion that phylogenetically older and histofunctionally less differentiated cells are later and more lightly

[196] C. D. Hughes, M. J. Swenson, G. K. Underbjerg, and J. S. Hughes, *Science* **116**, 252, (1952).

[197] B. I. Gol'dshtein, D. V. Vol'kenzon, L. G. Kondrateva, and N. D. Ul'yenova, *Biokhimiya* **15**, 173 (1950).

[198] F. Hansen, *Anat. Anz.* **16**, 417 (1899).

[199] F. Mall, *Am. J. Anat.* **1**, 329 (1902).

[200] M. R. Lewis, *Carnegie Inst. Wash. Contrib. Embryology* **6**, 45 (1917).

struck by the deficiency of antiscorbutics. . . . All these cells seem in the absence or deficiency of antiscorbutic to be subject to atrophy and yield a product which is quantitatively as well as qualitatively inferior. . . . If antiscorbutic is altogether lacking in the food, these cells go on living for a certain time and yield during this time products which deteriorate day by day till the activity is completely arrested and the death of the cell ensues."

A recent study of the morphogenesis of collagen fibers as observed in cultures of chick embryo skin by Porter[33] has furnished convincing evidence that the collagen fibers do form at the surfaces of cells. He found that fiber formation does not proceed at random in all directions as in the formation of fibrin in clot formation or as might be expected if collagen fibers polymerized out of an intercellular matrix. It appeared rather that "the fiber mat had been organized by cells, which, in migration, had deposited new fiber arrays above or below those already formed." In later studies, using a culture medium (Locke bouillon) without fibrin or serum components, he followed the stages in fiber production and observed that most of the ectoplasmic portion of the cell appeared to be involved in fiber production. He noted that "the collagen differentiates out of the surface, through the cell, and then will pull off the cell as a bundle of collagen fibrils, unit fibers." After leaving the cell as small striated fibrils 200 A. to 300 A. in diameter, they appeared to increase in diameter to 500 A. or 600 A. Porter considers it reasonable to assume that some of the collagen from the surface of the cell may go into solution and may later condense on the fine fibrils. Thus, the fundamental periodicity of the fiber is laid down by the cell, and with the pattern thus established additional units can be laid down outside the cell. Although in the Locke medium he did not obtain mature collagen, there was evidence that every third of the striae would expand to give the 640 A. periodicity characteristic of collagen.

(2) *Directly on the Extracellular Material.* According to this theory the fibroblasts would be able to form the supporting substances such as collagen but some colloid cement substance would be lacking. This hypothesis, advanced by Aschoff and Koch,[30] has been strongly supported experimentally by Wolbach and Howe[8] and Mazoué,[44] and with somewhat less conviction by Dalldorf[54] and Hunt.[53] In describing the cementing together of the fibrils as similar to the setting of gel, Dalldorf states that "it is precisely this phase of the formation of intercellular materials which may be completely controlled by vitamin C. Thus in guinea pigs which have been depleted of vitamin C, the ground substance and fibrils are present as in health but fibrils of collagen are not formed. When the deficiency is satisfied, translucent bundles or masses of collagenous materials reappear within 18 hours." Supporters of this theory hold that this period of time is too short for an appreciable amount of formation of completely new intercellular sub-

stances. They contend that the production of collagen would occur more rapidly than it would in the healing wounds of normal animals because of the accumulation of unset gel material. Ham and Elliott[98] maintained, however, that in the repair of skeletal tissues a completely new bone matrix can form within 48 hours, and they stated also that they found no histological evidence to support the contention that the new dentine which forms after administration of antiscorbutics represents the gelation of a previously produced unset gel. They referred to the fact that Fish and Harris[104] found no accumulation of fluid between the pulp and dentine which would have occurred had an unset gel been formed continuously during the scorbutic period. From these facts they conclude that the evidence for the gelation theory is inadequate.

c. Mode of Action

The chemical or physical means by which ascorbic acid exerts its controlling influence is not understood. It may function as a catalyst[27, 76, 85a, b 201, 202] or possibly as a component of other compounds.

(1) *Catalytic Action.* On the basis of experiments which have been conducted *in vitro* under conditions approximating those of the body, it would appear that the vitamin could act directly upon the cells, thereby conditioning them to secrete the enzymes, or it could act directly on the intercellular-matrix, creating conditions such as proper pH values and molecular arrangements which would enable the enzymes to function; also it might act by promoting conditions favorable to coupling processes such as establishing linkages between proteins and carbohydrates. Such an activity could be of particular importance at cell surfaces. The vitamin has in fact been shown to have an effect upon the surface activity of colloidal solutions by Keller and Künzel.[203] They found that under certain conditions such as high dilution and a close approach to physiological pH values ascorbic acid can alter surface active substances such as lecithin, either in the free form or when combined with tissue protein. Results of recent studies[204-206] also suggest the probability of a cell surface action of the vitamin. Harman and Warren[38] found that the cell walls in the embryos of scorbutic animals were indistinct and blurred in appearance whereas they were well defined in the corresponding tissues of normal animals indicating, differences in organization and/or in the products of synthesis at cell surfaces. The rounding of the special mesenchymal cells such as the fibroblasts is also an indication

[201] S. B. Wolbach and O. A. Bessey, *Physiol. Revs.* **22,** 233 (1942).
[202] B. Ekman, *Acta Physiol. Scand.* **8,** *Suppl.* 22, 196 pp. (1944).
[203] C. J. Keller and O. Künzel, *Z. ges. exptl. Med.* **103,** 704 (1938).
[204] F. Bernheim, M. L. C. Bernheim, and K. M. Wilbur, *J. Biol. Chem.* **174,** 257 (1948).
[205] M. E. Reid, *Am. J. Botany* **28,** 410 (1941).
[206] E. H. Newcomb, *Proc. Soc. Exptl. Biol. Med.* **76,** 504 (1951).

of a disturbance in the surface forces. Nungester and Ames'[121] finding of markedly reduced phagocytic activity and increased fragility of polymorphonuclear cells in scorbutic animals affords another illustration of abnormal cell surface behavior in scorbutic animals.

Lack of ascorbic acid is known to affect both the migration and arrangement of the intercellular matrix-producing cells and consequently also their products. Without it the arrangement is chaotic.[52] Danielli *et al.*[55] found that in scar tissue of guinea pigs receiving 2 mg. of ascorbic acid daily the orientation of fibroblasts is much more orderly than at levels of 0.7 mg. and 1.0 mg. This disarrangement of the matrix-producing cells in scurvy may be responsible for the amorphous structure of the secretory products in the different types of mesenchymal tissues. One of the best-known illustrations of this disorganizing effect is to be found in the odontoblasts of the teeth in which the cells lose their long, columnar shape, becoming shorter, rounder, and completely disarranged,[7] with the consequence that the secretory product, the dentine, loses its crystalline character.[104] Also, bone salts, which are normally laid down in crystalline form with a specific orientation of the molecules, are deposited as amorphous masses in scurvy.[49]

(2) *Ascorbic Acid or Its Derivatives As Components of Other Compounds.* (a) In Structural Materials. If the vitamin is used for structural purposes some modification of the molecule must occur, since connective tissue, for the production of which it is so essential, has itself an especially low ascorbic acid content. A significant approach to the problem has been made by Burns *et al.*[23] They fed the vitamin with labeled C^{14} in the carboxy position to normal and scorbutic guinea pigs and found that 20 to 30 % of the injected dose appeared as respired CO_2 within 24 hours; 3 to 6 % of the total activity was found in the urine, and 0.7 % in the feces, making a total recovery of slightly more than 23 to 36 %. Radioactivity in the tissues was distributed approximately the same as was ascorbic acid with no marked differences in the amounts in normal and scorbutic animals when compared on a unit weight basis. In a test with an animal which had been maintained on a daily dosage of 2 mg. of ascorbic acid, then given 3.32 mg. of the C^{14} product with no additional ascorbic acid for the following 10 days, 66 % of the administered C^{14} was expired as CO_2 during this period and 22 % was excreted in the urine. The fact that the teeth had a higher specific activity than such organs as the adrenals, liver, and spleen suggests the possibility of fixation and concentration of the radioactive material. The suggestion that ascorbic acid may be the precursor of the carbonate in the teeth would seem to follow. It would be of interest to determine if the teeth have a higher relative content of the vitamin than the other tissues mentioned. No definite evidence was obtained of radioactivity in the chondroitin

sulfate or collagen fractions isolated from cartilage and skin. Essentially all the C^{14} found in the nasal septum was present as ascorbic acid. In experiments with rats, Jackel *et al.*[24] used C^{14} glucose labeled uniformly in all positions and employed chloretone as a stimulant to ascorbic acid synthesis. The total transfer of C^{14} from glucose to ascorbic acid in 24 hours was about equal to the total conversion of administered carbohydrate to to ascorbic acid. It appeared that the glucose carbon chain was used in ascorbic acid synthesis without changing the ratio of activity in positions 1 or 2 to the total activity.

A number of investigators have suspected that ascorbic acid may be concerned directly with the synthesis of glucuronic acid. A recent study of the problem by Mosbach and King[207a] indicates, however, that the vitamin is probably not the precursor of this substance.

The results of these experiments are of considerable interest and appear to indicate that ascorbic acid is not used as such or in a modified form, at least in appreciable amounts, in the building of structural elements in cells.

(b) As a Component of an Enzyme. Evidence that ascorbic acid may function as a coenzyme in the oxidation of tyrosine has been presented by Sealock and coworkers[207b, 207c] and is discussed in the sections on metabolism and respiration (pp. 312, 333).

2. Relation to Growth

a. In Plants

Direct proof of a growth relation of ascorbic acid in green plants is difficult because it is always present in metabolically active tissues, since, like the rat, they have the capacity to synthesize it. Unfortunately there is no equivalent of the guinea pig in the realm of green plants. The highest content of the vitamin is found in regions of highest metabolic activity as in the leaves, in certain stages of some of the floral organs, and in the growing regions of shoots and roots. The concentration in regions of high metabolic activity suggests a possible function in growth. A number of investigators have found that very weak concentrations of ascorbic acid in the nutrient medium have a stimulatory effect on germination, but, on the other hand, some have obtained negative results.[208-212] Experiments conducted by

[207a] E. H. Mosbach and C. G. King, *J. Biol. Chem.* **185,** 491 (1950).
[207b] R. R. Sealock and R. L. Goodland, *Science* **114,** 645 (1951).
[207c] R. R. Sealock, R. L. Goodland, W. N. Sumerwell, and J. M. Brierly, *J. Biol. Chem.* **196,** 761 (1952).
[207d] Clegg, R. E., and Sealock, R. R., *J. Biol. Chem.* **179.** 1037 (1949).
[208] L. Havas, *Nature* **136,** 435 (1935).
[209] S. von Hausen, *Nature* **136,** 516 (1935).
[210] W. Davies, G. A. Atkins, and P. C. B. Hudson, *Ann. Botany (London)* [N.S.] **1,** 329 (1937).

Bonner and Bonner[211] suggest a possible explanation for this discrepancy. They found that certain varieties of peas with a naturally low concentration of ascorbic acid in the seedlings grew faster when additional vitamin was supplied whereas those types with a higher content, resulting from an inherent capacity for more rapid synthesis, did not respond to an additional supply. In studies with cowpea seedlings[213] it was found that a rough parallelism exists between growth and the ascorbic acid content of all the seedling organs whether they were grown in light or in darkness. Suggestive evidence of a relation of ascorbic acid to cell growth was obtained[205] in studies of some of the physical and chemical changes occurring in successive zones of the growing region of the primary root of cowpea seedlings. Determina-

TABLE VIII

Relative Values in Cells of Different Ages in Root Tips of Cowpea Seedlings[205]

Region	Volume	Dry weight	Surface area		Vtamin C	Vitamin C in dry material	Vitamin C in fresh weight
			As measured	Theoretical value			
Cell division	1	1	1	1	1	1	1
Cell division and elongation	3.2	2.20	2.34	2.2	2.62	1.20	0.80
Elongation	14.1	4.92	7.28	8.2	7.90	1.60	0.55
Elongation and maturation	35.1	8.77	11.8	13.8[a]	11.8	1.34	0.33
Maturation	37.6	9.09		13.8[a]	11.3	1.25	0.30

[a] These values are slightly high because no allowance was made for intercellular spaces.

tions were made of the ascorbic acid, nitrogen, and phosphorous content of the cells at different stages of development. It was observed that the ascorbic acid of a cell increased greatly during the transition from the embryonic state until approximately the time of completion of elongation. No appreciable increase was found after elongation ceased and secondary thickening of the wall began. Table VIII summarizes the results expressed as relative values per cell, the average value for a cell in the embryonic region being taken as unity. Continuous increases in volume, water, dry matter, nitrogen, and phosphorus per cell occurred during the transition from the embryonic to the mature condition. The change in surface area was the only one of the various factors studied in which the increase closely paralleled that of the vitamin. The relatively high content of ascorbic acid

[211] J. Bonner and D. Bonner, *Proc. Natl. Acad. Sci.* **24**, 70 (1938).
[212] B. Lustig and H. Wachtel, *Biochem. Z.* **297**, 386 (1938).
[213] M. E. Reid, *Am. J. Botany* **25**, 701 (1938).

per unit of dry matter in the region of expansion was an outstanding feature of the observations. The dry substance in the region of elongation contained 60 % more ascorbic acid than that of the embryonic region where nuclear activity is high and 30 % more than the region of maturation where cellulose accumulation appears to be the dominant activity. The data suggest that the increase in ascorbic acid is associated with an increase in the cytoplasm and/or cell wall. The parallelism between ascorbic acid and surface area is probably not fortuitous, and there may even be a true growth-controlling relationship between them.

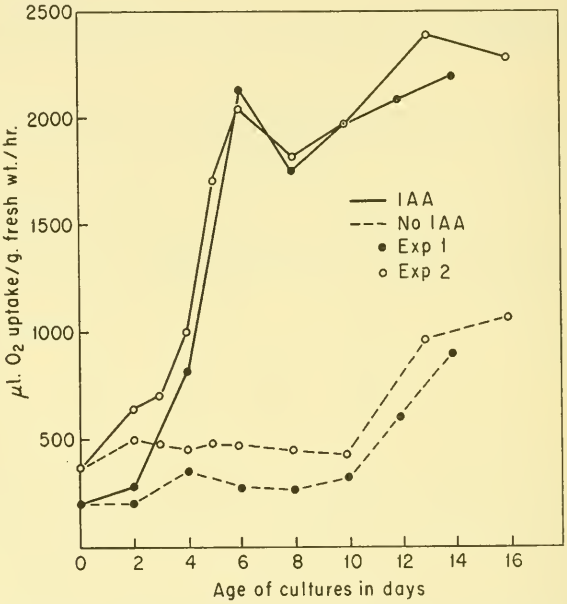

FIG. 2. Effect of 3.5 mg. per liter of indoleacetic acid on ascorbic acid oxidase activity of tobacco pith sections cultured *in vitro*. Curves based on oxygen consumption by homogenates on ascorbate. (By E. H. Newcomb.[206])

The concept of ascorbic acid as having a possible controlling effect on cell enlargement has received strong support from an interesting study by Newcomb[206] on the effects of auxin on the growth of cultured pith cells of tobacco stems. He found that the auxin caused a striking increase in the ascorbic acid oxidase activity of the tissue. This increase was followed by cell enlargement, gains both in fresh and dry weight, some respiratory increase, but no cell divisions. By differential centrifugation the ascorbic acid oxidase activity was found to be localized in the cell wall fraction; hence it was suggested that the enzyme occurs in the cytoplasm intimately associated with the wall where it may be causally related to growth. Figure 2 shows the quantitative results of these experiments. Newcomb suggested

that the ascorbic acid oxidase system may serve to generate energy-rich phosphate by the oxidation of phosphoascorbate or it may "alter the properties of the plasma membrane by increasing the potential difference across the membrane. This could be accomplished by maintenance of ascorbate in the oxidized form at the cell surface if it is largely in the reduced form in the cell interior. It is also conceivable that this enzyme plays some specific, though as yet unknown role in the organized, active growth of the primary wall which may control cell enlargement."

b. In Animals

In the absence of ascorbic acid in the diet, very young guinea pigs succumb so suddenly that a retarding effect on growth is often not observable.[214] Anderson and Smith[215] were the first investigators to eliminate inanition as a factor in the weight differences of animals receiving, as compared to those not receiving, ascorbic acid. In paired-feeding experiments they showed that guinea pigs given antiscorbutics (vitamin C had not been identified at this time) and whose food consumption was isocaloric with that of scorbutic animals attained a greater weight. Cohen[216] demonstrated that with a deficiency of ascorbic acid in the diet of guinea pigs there is first either a loss in weight or a failure to make a satisfactory gain in weight, then a failure to increase in length, and finally a failure to reach maturity. McHenry *et al.*[217] studied the effect of the pure vitamin on the weights of guinea pigs using the paired-feeding method and found that the animals receiving ascorbic acid were significantly heavier than those given only the basal diet. Since the differences in weight could not be caused by differences in food consumption, the suggestion was made that the lack of ascorbic acid had caused alterations in metabolism, water balance, and food absorption. They found that the appetite could be maintained by as little as 0.1 mg. of ascorbic acid per day. In a continuation of these studies Sheppard and McHenry[218] found that lack of ascorbic acid caused a diminished retention of water, which largely accounts for the observed differences in body weight. They also noted that the deficient animals in the paired-feeding groups retained considerably more body fat.

Because of the low requirement of ascorbic acid for the maintenance of appetite, it is not difficult to conduct growth tests with minute doses of the vitamin. In fact this method has been used by several investigators as a means of assaying for the vitamin in foodstuffs. Dunker *et al.*[219] conducted

[214] B. Cohen and L. B. Mendel, *J. Biol. Chem.* **35**, 425 (1918).
[215] W. E. Anderson and A. H. Smith, *J. Biol. Chem.* **61**, 181 (1924).
[216] M. B. Cohen, *Am. J. Diseases Children* **60**, 636 (1940).
[217] E. W. McHenry, E. J. Reedman, and M. Sheppard, *Biochem. J.* **32**, 1302 (1938).
[218] M. Sheppard and E. W. McHenry, *Biochem. J.* **33**, 655 (1939).
[219] C. F. Dunker, C. R. Fellers, and W. B. Esselen, *Food Research* **7**, 260 (1942).

weight response tests with ascorbic acid in daily doses ranging from 0.2 to 0.8 mg. per animal.

The relation of ascorbic acid to growth of specific organs, such as the teeth and adrenals, is discussed in the section on requirements (p. 380).

There is little evidence of a direct relation between ascorbic acid and growth of cells in animal tissues. Glick and Biskind[220] observed that the size and vitamin C content of the cells of the adrenal cortex increased regularly from the fetal to the adult stage of the animal. Studies of localization of the vitamin in cells of animal tissues reveal a distribution similar to that observed in plant cells, namely that it is found in cytoplasm, apparently often at the surface of the cells but none in the nucleus.

3. Distribution in Organs and Tissues

Ascorbic acid is probably present in all tissues of higher animals, at least during the developmental stages. Under normal conditions of nutrition tissues of young, rapidly growing animals tend to have a higher content than those of older animals fed the same diet.[221] The content tends to be highest in glandular tissues and lowest in fat, skin, connective tissue in general, and muscles. The adrenals, hypophysis,[222] and young thymus have a high content. Somewhat lower concentrations are found in spleen, pancreas, liver, testes, ovaries, brain, thyroid, and submaxillaries.[221] Table IX shows the average values found in guinea pig organs as determined by several investigators.[221, 223-226] A fairly high content of the vitamin has also been shown to be present in the walls of the intestinal tract.[227-229] Zilva[229] showed that in guinea pigs fed a diet containing ascorbic acid the vitamin is selectively absorbed by the intestinal walls. An accumulation in the intestinal wall is also produced when the vitamin is injected directly into the blood stream. With as much as 100 mg. injected the concentration was approximately the same as with ingestion of the normal diet containing the vitamin. With 70 mg. injected the concentration in the intestinal wall was only half as much. It is quite probable that with the very high injected dose the vitamin was being excreted into the lumen of the intestine.[226, 230] Ludany

[220] D. Glick and G. R. Biskind, *J. Biol. Chem.* **115**, 551 (1936).
[221] O. A. Bessey and C. G. King, *J. Biol. Chem.* **103**, 687 (1933).
[222] H. A. Salhanick, L. G. Zarrow, and M. X. Zarrow, *Endocrinology* **45**, 314 (1949).
[223] J. H. Roe, J. M. Hall, and H. M. Dyer, *Am. J. Digest. Diseases* **8**, 261 (1941).
[224] C. A. Kuether, I. R. Telford, and J. H. Roe, *J. Nutrition* **28**, 347 (1944).
[225] J. R. Penney, S. S. Zilva, *Biochem. J.* **40**, 695 (1946).
[226] M. E. Reid, *J. Nutrition* **42**, 347 (1950).
[227] E. Harde and J. Wolff, *Compt. rend. soc. biol.* **116**, 288 (1934).
[228] F. G. Hopkins, *J. Soc. Chem. Ind.* **53**, 874 (1934).
[229] S. S. Zilva, *Biochem. J.* **29**, 100 (1935).
[230] M. E. Reid, *Proc. Soc. Exptl. Biol. Med.* **68**, 403 (1948).

and Zselyonka[231] also studied the content of vitamin C in the tissues of the gastrointestinal tract. They found a content of 38 mg. % in the mucosa of the small intestine. In the stomach, the mucosa of the fundus contained more than that of the pylorus.

In the nervous system both the tissues and their bathing fluids contain appreciable amounts of ascorbic acid. The content in the human brain has been reported to range from 13 to 26 mg. %[232] and that in the peripheral nerves was found to be approximately one-fourth as much. The content in the spinal fluid varies with that of the plasma and is about four times as

TABLE IX

AVERAGE CONTENT (MG.%) OF ASCORBIC ACID IN GUINEA PIG TISSUES

Investigators	Bessey and King (1933)[221]	Roe, Hall and Dyer (1941)[223]	Kuether, Telford, and Roe (1941)[224]	Penney and Zilva (1946)[225]	Reid (1950)[226]
Diet used	Sherman basal diet + spinach	Rabbit chow + cabbage	Prepared diet + ascorbic acid	Mixed diet + cabbage	Prepared scorbutic diet 10 hr. after ascorbic acid injection
Method of assay	Indophenol	Indophenol	Osazone	Osazone	Osazone
Liver	10	37	32.8	16.7	37.5
Adrenal gland	75	336	166	150	178
Spleen		42	41.9	43	74
Kidney	8.7		11.6	8.5	15.9
Brain	14	24.5	22.8		
Skeletal muscle	3.2		3.12	3.1	
Heart	8.8		8.98		
Whole blood	2.0		1.16	0.75	1.36
Testes	18				

high.[233] A direct relation of the vitamin to physiological function of the nervous system has not been established, although there is some evidence[89] that it affects the formation of cerebrosides. Also, it has been observed to catalyze the oxidation of phospholipids in suspensions of brain tissue.[234, 235]

Variations in content of the vitamin within the tissues of certain glandular organs have been observed. The intermediate and anterior lobes of the hypophysis, the adrenal cortex, corpus luteum, and interstitial tissue exceed all other tissues of the body in their vitamin C content. Of particular

[231] G. Ludany and L. Zselyonka, *Biochem. Z.* **294**, 108 (1937).
[232] J. Melka, *Arch. ges. Physiol.* **237**, 216 (1936).
[233] F. Westergaard, *Nord. Med.* **16**, 3536 (1942); *Ber. ges Physiol.* **133**, 644 (1943).
[234] K. A. C. Elliott and B. Libet, *J. Biol. Chem.* **152**, 617 (1944).
[235] F. Bernheim, K. M. Wilbur, and D. B. Fitzgerald, *J. Gen. Physiol.* **31**, 195 (1947).

interest is the relation between the ascorbic acid content and state of activity of the organ. Giroud *et al.*[236] reported finding 17 mg. % in cow ovaries without corpora lutea, and in the corpora lutea there was considerably more. The maximum found was 103 mg. % in the fully developed periodic corpus luteum and 162 mg. % in the corpus luteum of pregnancy. During regression of the corpus luteum the ascorbic acid content was found to decrease. Other reports have supported these findings.[220, 237] Similarly, in the testes an especially high content has been found in the interstitial tissue by the use of histochemical methods.[238] Glick and Biskind[220] found 21 to 30 mg. % in the entire glands whereas in the interstitial tissue the content was four to twelve times this amount. In both the ovary and testes there appears to be a relationship between the high content and state of activity of the tissue. A connection with hormonal functions is suggested.

Roe *et al.*[223] made a study of the relation of ascorbic acid to gastric function. Guinea pigs maintained on a scorbutigenic diet were used. Histamine was injected every 15 minutes into a fasted animal, and at half-hour intervals for a period of 2 hours the stomach contents were removed, and the volume, total acidity, and free hydrochloric acid determined. Up to the time of onset of severe scurvy symptoms there was little change in secretory function, but with the development of acute symptoms there was a marked reduction in volume of secretion and a decrease in acidity.

4. Relation to Metabolism

Several investigations have shown diurnal fluctuations in the ascorbic acid content of rapidly growing plants, with the vitamin increasing during the day and decreasing at night.[239-243a] The loss in ascorbic acid content is associated with growth. If, however, the plants are kept in darkness at temperatures too low for growth, a loss of ascorbic acid does not occur.[243b] These nightly losses of the vitamin may be chiefly, but not necessarily, caused by respiratory processes similar to the respiratory losses of this substance in animals as shown by Burns *et al.*[23]

A close relation exists between growth and the ascorbic acid content of

[236] A. Giroud, I. Cesa, R. Ratsimamanga, and M. Rabinowicz, *Compt. rend. soc. biol.* **122**, 899 (1936).

[237] A. A. Policard and M. Ferrand, *Compt. rend. soc. biol.* **123**, 1081 (1936).

[238] E. Tonutti, *Z. Vitaminforsch.* **9**, 349 (1939).

[239] H. G. Moldtmann, *Planta* **30**, 297 (1939).

[240] M. E. Reid, 134th Meeting, American Association for the Advancement of Science, Milwaukee, Wisconsin, 1939.

[241] E. F. Kohman and D. R. Porter, *Science* **92**, 561 (1940).

[242] A. M. Smith and J. Gillies, *Biochem. J.* **34**, 1312 (1940).

[243a] M. E. Reid, *Bull. Torrey Botan. Club* **68**, 359 (1941).

[243b] M. E. Reid, *Bull. Torrey Botan. Club* **68**, 519 (1941).

tissues in animals much as in plants. The disappearance of the vitamin is greater during periods of rapid growth. In the guinea pig, supplied daily by injection with constant amounts (5 mg. per 100 g. of body weight) of ascorbic acid, less of the vitamin in the urine is excreted per unit of body weight by the young than by the full-grown animal, and the younger the animal and the more rapidly it grows, the smaller the amount excreted.[244] Part of the administered dose is held by the tissue, some is destroyed and is accounted for chiefly as carbon dioxide, possibly some as oxalic acid in the feces, and the remainder is excreted in the urine. The metabolic losses of the vitamin may be due entirely to breakdown to carbon dioxide, oxalic acid, and possibly other products.[23] However, as pointed out previously, the complete picture of the biochemical metabolism of ascorbic acid has not been revealed.

a. Proteins and Amino Acids

Ascorbic acid plays an important role in intermediate protein metabolism, particularly with respect to the oxidation of the aromatic amino acids, tyrosine and phenylalanine. The literature on the subject has recently been reviewed by Woods.[245] When the aromatic amino acids are fed in large amounts to premature infants subsisting on cow's milk[246] or to guinea pigs on a diet lacking ascorbic acid, there is an excretion of abnormal metabolites (*p*-hydroxyphenyllactic and *p*-hydroxyphenylpyruvic acids) in the urine.[90] Sealock *et al.*[90] showed also that guinea pigs receiving supplements of tyrosine or phenylalanine or their derivatives, and only maintenance amounts of ascorbic acid, excreted homogentisic acid in amounts proportional to the intake of ascorbic acid. Of a large series of phenylalanine and tyrosine derivatives only L-phenylalanine, L-tyrosine, and phenylpyruvic acid showed any dependence on the intake of ascorbic acid. The vitamin appeared to function at a point prior to the formation of the tyrosine keto acids.[247] Investigations by Knox and LeMay-Knox[248] have revealed the steps in the oxidation of tyrosine in liver homogenates. The initial reaction is a tyrosine-α-ketoglutarate transamination with the formation of glutamate and *p*-hydroxyphenylpyruvate. Addition of ascorbic acid to the mixture had no effect on the rate of deamination but affected notably the oxidation of *p*-hydroxyphenylpyruvate. The action of ascorbic acid appeared to be chiefly one of continuing the oxidation of tyrosine to the formation of acetoacetate. These investigators reported also that isoascorbic acid could replace

[244] M. E. Reid, *J. Nutrition* **35**, 619 (1948).
[245] R. Woods, *Borden's Rev. Nutrition Research* **10**, No. 7 (1949).
[246] S. Z. Levine, E. Marples, and H. H. Gordon, *Science* **90**, 620 (1939).
[247] D. H. Basinski and R. R. Sealock, *J. Biol. Chem.* **166**, 7 (1946).
[248] W. E. Knox and M. LeMay-Knox, *Biochem. J.* **49**, 686 (1951).

ascorbic acid with a similar effect. Painter and Zilva[249] in *in vitro* experiments reported essentially the same results with D-glucoascorbic acid as with equivalent amounts of ascorbic acid, which tended to throw doubt on the effect as being one of vitamin action. Rienits[250a] published data, however, which indicated that L-ascorbic acid is specific and that D-ascorbic acid and D-glucoascorbic acid had no effect. He showed by oxygen consumption measurements and by determination of the disappearance of hydroxyphenyl groups from liver slices that oxidation of tyrosine is related to the ascorbic acid nutrition of the animal.

The discrepancy in the response of liver slices and liver homogenates in the oxidation of tyrosine is at least partially explainable by results reported by Sealock's laboratory.[207b, c, d] Sumerwell and Sealock[250b] showed that a considerably greater proportion of the ascorbic acid in the livers of scorbutic animals, as compared to that in normal animals, is present in bound form and much of it is not measured by the usual methods of assay. More recently, Sealock *et al.*[207c] have shown that when the ratio of tissue to tyrosine is large, as in the case of homogenates, sufficient ascorbic acid is present even in scorbutic livers to cause oxidation of the tyrosine, whereas in the liver slices the amount of the vitamin present is too small to oxidize the tyrosine. Their results also showed that for completing the oxidation of tyrosine the vitamin is needed only in extremely small amounts. Studies with glucoascorbic acid, D-isoascorbic acid, and reductone, compounds related chemically and structurally to ascorbic acid, gave indication that it is the enediol linkage which is responsible for the action of the vitamin as a coenzyme in tyrosine oxidation. These are not compounds normally present in living tissues. One of them, glucoascorbic acid, has been shown[251a] to produce a slight alleviation of scurvy symptoms when fed to animals subsisting on a scorbutigenic diet. There are no ready explanations for this type of response, but it would seem that the possibility should not be overlooked that liver tissue may contain a factor which aids in the conversion of a small amount of the compound to the L-ascorbic acid form.

Recent results of Schepartz and Nadel[251b] with acetone powder preparations of guinea pig liver have demonstrated that the failure in tyrosine metabolism in scorbutic animals, as indicated by a decrease in enzymatic activity, is a result of vitamin C deficiency and not due to inanition.

Woodruff and Darby[252] showed that folic acid as well as ascorbic acid can prevent the excretion of abnormal metabolites in scorbutic guinea pigs.

[249] H. A. Painter and S. S. Zilva, *Biochem. J.* **46**, 542 (1950).
[250a] K. G. Rienits, *J. Biol. Chem.* **182**, 11 (1950).
[250b] W. N. Sumerwell and R. R. Sealock, *J. Biol. Chem.* **196**, 753 (1951).
[251a] W. G. Shafer, *J. Dental Research* **29**, 831 (1942).
[251b] B. Schepartz and E. M. Nadel, *Federation Proc.* **11**, 425 (1952).
[252] C. W. Woodruff and W. J. Darby, *J. Biol. Chem.* **172**, 851 (1948).

These results were confirmed by Rienits.[250a] Salmon and May[253] obtained somewhat different results with folic acid in monkeys. They found no difference between normal and scorbutic animals in the urinary excretion of *p*-hydroxyphenyl compounds and keto acids. After administration of 2 g. of tyrosine by gavage daily for a prolonged period, a scorbutic animal excreted very large amounts of the compounds, but a normal animal put out only very small amounts. Administration of ascorbic acid to the scorbutic animal reduced the excretion of the compounds to the level of the normal animal, but folic acid had no effect.

Vitamin C may play some role in detoxification of certain amino acids. This has been implied by the work of De Caro and Bertrami,[254] who found that 10 mg. of ascorbic acid daily were required to counteract the effect of 0.1 g. of cystine added daily to the scorbutigenic diet. In the absence of adequate amounts of ascorbic acid the addition of cystine shortened the survival time of guinea pigs. Rygh[255] has reported also that administration of L-cystine hastens the development of scurvy. Although no work has been done on the relation of ascorbic acid to methionine detoxification[256-258] in rats, this would appear to be a problem which should be investigated.

Christensen and Lynch[22] found a marked decrease of glycine and glutamine in the skeletal muscles and of glutamine in the liver of ascorbic acid-deficient guinea pigs as compared to normal animals or to fasted controls. Amino acids other than glycine or glutamine were increased in the deficient animals.

Murray[260] observed that normal animals had a much greater ability to convert DL-alanine to liver glycogen than did the scorbutic animals.

b. Carbohydrates

(1) *Absorption.* A study by Murray and Morgan[261] demonstrated a lower absorption after glucose ingestion in scorbutic than in normal guinea pigs. This work was based on analyses of the intestinal contents at various intervals after giving glucose. These findings were confirmed by Banerjee and Ghosh.[262]

(2) *Metabolism.* There has been no clear indication of a disturbance in

[253] R. J. Salmon and C. D. May, *J. Lab. Clin. Med.* **36**, 591 (1950).
[254] L. De Caro and W. Bertrami, *Quaderni nutriz.* **2**, 165 (1935).
[255] O. Rygh, *Bull. soc. chim. biol.* **33**, 133 (1951).
[256] D. P. Earle, K. Smull, and J. Victor, *J. Exptl. Med.* **76**, 317 (1942).
[257] K. A. J. Wretlind, *Acta Physiol. Scand.* **20**, 1 (1950).
[258] J. F. Van Pilsum and C. P. Berg, *J. Biol. Chem.* **183**, 279 (1950).
[260] H. C. Murray, *Proc. Soc. Exptl. Biol. Med.* **69**, 351 (1948).
[261] H. C. Murray and A. F. Morgan, *J. Biol. Chem.* **163**, 401 (1946).
[262] S. Banerjee and N. C. Ghosh, *J. Biol. Chem.* **180**, 189 (1949).

the oxidation of carbohydrates in scurvy.[263] In the over-all metabolism as shown by the basal metabolic rate and respiratory quotient, no difference in energy output has been found between normal and deficient animals.[261] Sylvest[264] reported finding no definite evidence of a relation between the ascorbic acid of the serum and the sugar tolerance curve. Frommel *et al.*[265] observed hyperglycemia during the first 5 days on the scorbutigenic diet followed by a hypoglycemic period which was progressive up to the death of the animal. Nair[266] reported the fasting blood sugar level to be the same in normal and scorbutic animals but glucose tolerance was lower in the scorbutic group. Sigal and King[267] found, however, that successive stages of ascorbic acid deficiency resulted in a corresponding rise in the fasting blood sugar level and a definitely lower glucose tolerance. Murray and Morgan[261] also obtained significantly higher blood levels in glucose tolerance tests with scorbutic as compared to normal animals, both groups of which had been fasted 24 hours and then were given orally 5 ml. of a solution containing 2.5% glucose. Grolitsch and Stöger[268] observed that the hyperglycemia which tended to occur after physical exertion in human subjects deficient in vitamin C was decreased or did not occur after administration of the vitamin.

Although the exact role of insulin in carbohydrate metabolism has not been determined, there is, nevertheless, some reason for believing that it has some effect on the over-all metabolism. Banerjee[269] studied the effect of ascorbic acid on the insulin content of the pancreas. Extracts made from the glands of normal and scorbutic animals were injected into rabbits, and changes in the blood sugar determined. The results indicated that the insulin content of the pancreas of the scorbutic animals was reduced to about one-eighth the normal value. He suggested that the change in carbohydrate metabolism in scurvy is due to a diminished insulin content of the pancreas. He found, however, that administration of ascorbic acid above an optimum level did not increase the amount of insulin in the pancreas.

(3) *Storage.* Without exception, investigators who have studied the relation of ascorbic acid to glycogen storage in the liver have found it to exert a stimulating effect. Altenburger[270] and Yoshinare[271] were among the

[263] L. Armentano, A. Bensath, A. Hamori, and A. Koranyi, *Z. ges. exptl. med.* **96**, 321 (1935).
[264] O. Sylvest, *Acta Med. Scand.* **110**, 183 (1942).
[265] E. Frommel, E. Aron, and J. Aron, *Arch. sci. phys. et nat.* **26**, *Suppls.* 58–62 (1944).
[266] K. R. Nair, *Ann. Biochem. Exptl. Med.* **1**, 179 (1941).
[267] A. Sigal and C. G. King, *J. Biol. Chem.* **116**, 489 (1936).
[268] K. Grolitsch and R. Stöger, *Klin. Wochschr.* **20**, 1166 (1941).
[269] S. Banerjee, *Ann. Biochem. Exptl. Med.* **4**, 33 (1944).
[270] E. Altenburger, *Klin. Wochschr.* **15**, 1129 (1936).
[271] T. Yoshinare, *Japan. J. Med. Sci. IV, Pharmacol.* **10**, Absts. 48 (1936).

first to study the problem. They found that glycogen deposition is decreased in the livers of guinea pigs on a scorbutigenic diet. Yoshinare observed a decrease in muscle glycogen also. Administration of the vitamin to deficient animals caused a prompt storage of glycogen. Ratsimamanga[272] observed the influence of the vitamin on the behavior of the animal at rest. In guinea pigs on a diet deficient in ascorbic acid, the liver and muscle glycogen decreased to approximately one-fifth the normal content whereas muscle and blood lactate tended to increase. The rates of return to normal were roughly proportional to the amount of ascorbic acid supplied. Several workers have found that injection of ascorbic acid into guinea pigs or rabbits increased the glycogen content of the liver.[261, 273-275] By the intravenous injection of 10 ml. of a 10% lecithin suspension per kilogram, Terada[274] obtained complete depletion of glycogen in rabbits after 12 hours. Two and five-tenths grams of glucose per kilogram were then given, and the animals were sacrificed after 3 hours by which time the absorption and storage of the major portion of the glucose had occurred. He found that subcutaneous administration of L-ascorbic acid (50 mg. per kilogram) increased the liver glycogen stores to 1.9%, which was 63% more than those of the control group. Doses of D-araboascorbic acid also increased the glycogen storage to 1.8% or 61% more than the control value. These experiments showed clearly that the glycogenic action of the two forms of ascorbic acid was the same but unrelated to their scurvy-preventing ability. The possibility of conversion of either of the two forms of ascorbic acid to glycogen was not suggested.

(4) *Summary of Relations of Ascorbic Acid to Carbohydrate Metabolism.* Present evidence appears to indicate that a tendency to lower glucose tolerance and a higher fasting blood sugar level are characteristic of scurvy in guinea pigs. Scorbutic animals show slower absorption after glucose ingestion than do normal animals. The insulin content of the pancreas is appreciably lowered in scurvy, but this is undoubtedly not the only defect in glucose metabolism. The complete relationship of ascorbic acid to glucose tolerance has not been elucidated.

c. Fats

(1) *Effect of Lack of Ascorbic Acid on Content of Tissue Lipids.* Although control of food intake, as should be expected, is especially important in studying these problems,[99, 180, 276] in several investigations which have been

[272] A. R. Ratsimamanga, *Travail humain* **7**, 303 (1939).
[273] A. Morelli and L. d'Ambrosia, *Arch. sci. biol.* **24**, 351 (1938).
[274] K. Terada, *Tohoku J. Exptl. Med.* **36**, 180 (1939).
[275] I. Watanabe, *Tohoku J. Exptl. Med.* **35**, 65 (1939).
[276] M. A. Spellberg and R. W. Keeton, *Proc. Soc. Exptl. Biol. Med.* **41**, 570 (1939).

reported, this procedure was not followed.[116, 179] Iwabuchi[116] and Nagayama and Tagaya[179] stated that the fat content of tissues is decreased in experimental scurvy in guinea pigs, particularly in the muscles and adrenals. Other investigators[261, 276] have also observed a reduction in body fat in scurvy. However, Tomlinson[277] found no definite abnormality in the lipid content of the liver, kidneys, or adrenals in experimental scurvy in monkeys. Beyer[278] observed little difference in the fat content or structure of the livers of scorbutic guinea pigs as compared to normal controls. Sadhu[89] found that phospholipid and choline values were not altered in scurvy. MacLean *et al.*[99] conducted a paired-feeding test to study the effect of inanition and scurvy on the tissues of guinea pigs. Microscopic examination revealed a normal condition of the livers in the scorbutic group, but determinations were not made of the fat content. Baldwin *et al.*[180] also used the paired-feeding technique and found no significant differences in the amounts or composition of the fat of the liver, adrenals, and carcass between scorbutic and normal guinea pigs. Another group of investigators have found an increase in liver fat in scurvy,[161, 180, 279] but it is possible that some factor other than lack of ascorbic acid may have been involved in producing the effect. More work needs to be done on fat metabolism in the guinea pig.

(2) *Cholesterol.* Mouriquand and Leulier[280] determined the cholesterol content of the blood and organs in experimental scurvy and found a marked decrease in the content in the adrenals but no significant changes in the other organs or in the blood. Randoin and Michaux[281] and Nagayama and Tagaya[179] reported that the amount of cholesterol in the adrenal capsule of scorbutic guinea pigs is less than in that of normal animals. The latter investigators found also that the amount of cholesterol in the lungs decreased markedly in scurvy. In the testes and muscles there was also a slight decrease in cholesterol. Bessey *et al.*[161] found that the most characteristic lesion in the organs of scorbutic guinea pigs was the depletion of fat and cholesterol from the cortex of the adrenals. Interest in the cholesterol content of the adrenals and the hormonal factors influencing it has been greatly stimulated by the investigations of Oesterling and Long.[182a] In paired-feeding experiments they have presented convincing evidence that in the early stage of scurvy the adrenal cholesterol increases but that it decreases markedly in the late stage of the disease. Sayers *et al.*[282] reported that injections of adrenotrophic hormone into rats and guinea pigs resulted in a

[277] T. H. Tomlinson, *Public Health Repts.* (*U. S.*) **57**, 987 (1942).
[278] K. H. Beyer, *Arch. Internal Med.* **71**, 315 (1943).
[279] A. Terbruggen, *Verhandl. deut. pathol. Ges.* **31**, 114 (1938).
[280] G. Mouriquand and A. Leulier, *Compt. rend. soc. biol.* **93**, 1314 (1925).
[281] L. Randoin and A. Michaux, *Compt. rend. soc. biol.* **183**, 1055 (1926).
[282] G. Sayers, M. A. Sayers, H. L. Lewis, and C. N. H. Long, *Proc. Soc. Exptl. Biol. Med.* **55**, 238 (1944).

quick fall in adrenal ascorbic acid and a slower fall in cholesterol whereas the levels of these substances in other tissues remained unchanged. These workers suggested that the release of adrenal cortical hormone may be involved in lowering the content of ascorbic acid and cholesterol in the adrenals. Active study of these problems is continuing in several laboratories.

(3) *Fatty Acid Oxidations.* Quastel and Wheatley[283] studied the effect of the vitamin on fatty acid oxidations in the liver and concluded that the vitamin is important in maintaining the metabolism of fatty acids at a normal level. Rusch and Kline[284] reported that ascorbic acid catalyzes the oxidation of phospholipids in the liver, and Elliott and Libet[234] found that the vitamin augmented the phospholipid stimulation of oxidation in suspensions of brain tissue. Bernheim, Wilbur, and Fitzgerald[285] reported that brain protein, also that of several other organs, is combined with a compound probably containing hydroxyl groups attached to a benzene ring. Ascorbic acid acted as a catalyst in the oxidation of the compound and in the reaction the protein was split off and aldehyde groups became exposed.

Abramson[286] measured the oxidation of unsaturated fatty acids in normal and scorbutic guinea pigs by means of the thiobarbituric acid color reaction of Kohn and Liversedge.[287] He observed that in scurvy the oxidation was decreased in brain, testes, medulla oblongata, adrenals, and kidneys but in spleen and heart there was little difference from the normal. Bernheim, Bernheim, and Wilbur[204] found that the color obtained upon addition of thiobarbituric acid to lecithins or compounds containing them are due to a product of the oxidation of unsaturated fatty acids such as linolenic. Ascorbic acid appeared to catalyze the oxidation of lecithin either in free form or when combined with tissue protein. In the reaction the oxidation could be followed quantitatively.

d. Minerals

The study of mineral metabolism in scurvy has not been given the attention it deserves. Some of the problems undoubtedly should be reinvestigated, using a dietary regime complete in all respects except for the lack of ascorbic acid. On the basis of blood studies it would appear that mineral metabolism is not greatly deranged in scurvy. This does not signify, however, that an increased turnover of minerals does not occur in the tissues.

(1) *Calcium.* It is impossible to state with any degree of finality whether

[283] J. H. Quastel and A. H. M. Wheatley, *Biochem. J.* **28**, 1014 (1934).
[284] H. P. Rusch and B. E. Kline, *Cancer Research* **1**, 465 (1941).
[285] F. Bernheim, K. M. Wilbur, and D. B. Fitzgerald, *J. Gen. Physiol.* **31**, 195 (1947).
[286] H. Abramson, *J. Biol. Chem.* **178**, 179 (1949).
[287] H. Kohn and M. Liversedge, *J. Pharmacol. Exptl. Therap.* **82**, 292 (1944).

ascorbic acid does[99, 234, 288-295] or does not[7, 150, 296-298] have any influence on calcium metabolism. Ruskin[299, 300] has suggested that an intimate relation exists between ascorbic acid and calcium and that calcium is probably absorbed from the intestine as calcium ascorbate. Daniels *et al.*[301] found no relation between the ingestion of 2.7 to 12.5 mg. of ascorbic acid and calcium retention per kilogram in children. Henry and Kon[302] found no effect of an addition of 2 mg. daily of ascorbic acid on the retention of calcium in rats kept on a diet low in calcium. Mallon and Lord[303] observed that addition of 5 ml. of lemon juice to young growing rats maintained on a diet low in calcium caused no increase or decrease in calcium retention. Fraser[304] found no evidence of aggravation of ascorbic acid deficiency in monkeys by superimposing a calcium deficiency upon it. The symptoms of vitamin C deficiency actually appeared to be delayed in animals subjected to the combined deficiency of the two factors. In histological studies of Frazier's animals, Tomlinson[277] found the changes very similar to those observed in the group with ascorbic acid deficiency only. However, hemorrhages did not occur quite so frequently and when present were usually not so extensive. The effects of excess calcium on the onset of deficiency symptoms with diets lacking ascorbic acid have not been reported but would be of considerable interest.

Lust and Klocman[305] found a positive calcium balance in human scurvy and a negative balance in the healing stage. Höjer[7] suggested the following as an explanation of the phenomenon: "In the early stages of scurvy a bone is formed which is calcified, but quantitatively as well as qualitatively inferior. The resorption of the old bone seems to proceed normally. The

[288] G. Toverud, *J. Biol. Chem.* **58**, 583 (1923).
[289] B. W. Fairbanks and H. H. Mitchell, *J. Nutrition* **11**, 551 (1936).
[290] J. C. Aub, *J. Am. Med. Assoc.* **109**, 1276 (1937).
[291] H. Lucke and E. Heckmann, *Arch. Exptl. Pathol. Pharmakol.* **189**, 87 (1938).
[292] H. Lucke and J. Wolf, *Arch. Exptl. Pathol. Pharmakol.* **189**, 628 (1938).
[293] C. S. Lanford, *J. Biol. Chem.* **130**, 87 (1939).
[294] C. S. Lanford, *J. Nutrition* **23**, 293 (1942).
[295] M. L. Shepherd, I. G. Macy, H. A. Hunscher, and F. C. Hummel, *J. Pediat.* **16**, 704 (1940).
[296] M. Goettsch and K. M. Key, *Quart. J. Pharmacol. Exptl. Therap.* **1**, 168 (1928).
[297] H. Kapp and A. Schetty, *Biochem. Z.* **290**, 58 (1937).
[298] E. N. Todhunter and W. Brewer, *Am. J. Physiol.* **130**, 310 (1940).
[299] S. L. Ruskin, *Am. J. Digest. Diseases* **5**, 408 (1938).
[300] S. L. Ruskin and R. Jonnard, *Am. J. Digest. Diseases* **5**, 676 (1938).
[301] A. L. Daniels, G. J. Everson, O. E. Wright, M. F. Deardorff, and F. I. Scoular, *J. Nutrition* **14**, 317 (1937).
[302] K. M. Henry and S. K. Kon, *Biochem. J.* **33**, 1652 (1939).
[303] M. G. Mallon and D. J. Lord, *J. Am. Dietet. Assoc.* **18**, 303 (1942).
[304] H. F. Fraser, *Public Health Repts (U. S.)* **57**, 959 (1942).
[305] F. Lust and L. Klocman, *Jahrb. Kinderheilk* **75**, 663 (1912).

new bone is of small scale and cannot absorb the quantity of calcium made free from the old bone. There ensues in the body a surplus of calcium, and a precipitate excretion takes place. . . . Afterwards, in the progress of scurvy a circumstance arises which acts in the opposite direction. Different parenchymal organs get more and more atrophic and necrotize partially. Dying tissue generally has greater affinity for calcium and thus we see in scorbutic guinea pigs calcifications appear to a large extent in different organs, principally muscles and liver. This may explain the calcium balance being positive during this stage. In healing, again a strong excretion sets in which has been noted by several investigators."

Another aspect of the problem of interrelations of ascorbic acid and calcium is shown in connection with toxic doses of these substances. Valenzuela[306] studied the problem by injecting large doses of ascorbic acid intravenously into mice. The administration of 3.75 mg. per gram caused death immediately. By perfusing frog hearts with Ringer's solution containing up to 0.8 or 0.9% ascorbic acid he observed a decrease in the amplitude and frequency of the contractions with a decrease in the minimum volume and final arrest in the diastole. Addition of calcium chloride or calcium gluconate reduced the toxicity of the ascorbic acid, and similarly ascorbic acid increased the tolerance against large doses of calcium salts injected intravenously. The evidence of a specific effect of ascorbic acid as a cause of the toxicity is not convincing, since comparable tests were not made with other types of acids.

The interrelations of calcium and ascorbic acid with respect to cell surfaces and intercellular substances were reviewed and discussed in 1943.[307] The special need of the vitamin by those cells in which calcium metabolism is very active is not without significance. Chambers' studies[308, 309] showed that repair of injury to cells is impossible in the absence of calcium because of inability of the protoplasm to form a new interface. That ascorbic acid also plays a role in the formation of the cell boundary is shown by Harman and Warren's finding[38] of indistinct cell walls in the tissues of embryos of scorbutic animals. It seems quite possible that the vitamin acts in conjunction with calcium to produce coagulative changes in cell surfaces in both plant and animal tissues and that the intercellular cement may be an end product of this action. That the vitamin functions also in the maintenance of the wall is shown by the disappearance of cell boundaries in scorbutic tissues as observed by Höjer[7] and Meyer.[88] Eichelberger *et al.*[310] studied the

[306] M. A. Valenzuela, *Farmacoterap. actual* (*Madrid*) **2**, 712 (1945).
[307] M. E. Reid, *Physiol. Revs.* **23**, 76 (1943).
[308] R. Chambers, *Am. Naturalist* **72**, 141 (1938).
[309] R. Chambers, *Cold Spring Harbor Symposia Quant. Biol.* **8**, 144 (1940).
[310] L. Eichelberger, F. C. McLean, and A. Catterall, *J. Biol. Chem.* **142**, 467 (1942).

distribution of calcium between the cells and extracellular fluids of the skeletal muscles and liver in dogs and concluded that, unless some of the calcium is within the cells, a part of it must be in an un-ionized combination with some extracellular substance other than the protein of the extracellular fluid. They did not suggest, however, the possibility of a linkage of a portion of the calcium with a constituent of an intercellular cement substance to form a difficultly soluble compound. In tests with radioactive ascorbic acid, Burns *et al.* recently reported[23] finding a higher specific radioactivity in the teeth than in organs such as the adrenals. Their results suggest fixation and concentration of the radioactive material in the teeth, but it seems doubtful that all the material could be ascorbic acid as such, however. The finding in the teeth of radioactive carbon originating from ascorbic acid suggests a relation between the metabolism of calcium and ascorbic acid. It seems possible that ascorbic acid may be transported as calcium ascorbate to calcification areas where it is, at least in part, metabolized to carbon dioxide and deposited as carbonate.

(2) *Phosphorus.* Newcomb[206] suggested that the ascorbic acid-oxidase system in plant tissues may serve to generate energy-rich phosphate by the oxidation of phosphoascorbate, a substance which has not been identified as a constituent of either plant or animal tissues. In scorbutic animals changes in phosphorus metabolism may be entirely secondary to the changes in calcium. Some workers have found that disturbances in phosphorus metabolism in scurvy are of a relatively low order[150] whereas others have reported a fall in blood phosphate in scurvy.[311-313] Ascorbic acid appears to play a part in the formation or stabilization of alkaline phosphatase, an enzyme, the level of which is reduced markedly in severe scurvy.[55, 298, 314-319] Bourne[316] suggested that "one of the functions of vitamin C is to allow the production of a phosphatase-impregnated bone matrix upon which bone salt is immediately deposited."

Horvath and Tebbe[320] found only minor variations in the concentration of the various phosphorus compounds in the gastrocnemius muscle of scorbutic guinea pigs during the first 16 days on the deficient diet. With the onset of deficiency symptoms following this period, the levels decreased

[311] H. von Euler and K. Myrbäck, *Hoppe-Seyler's Z. physiol. Chem.* **148,** 180 (1925).
[312] H. Leone, *Riv. clin. pedia.* **32,** 1185 (1934).
[313] R. Matricardi, *Riv. clin. pediat.* **36,** 351 (1938).
[314] H. Schwachman and B. S. Gould, *J. Nutrition* **23,** 271 (1942).
[315] B. S. Gould and H. Schwachman, *Am. J. Physiol.* **135,** 485 (1942).
[316] G. H. Bourne, *J. Physiol. (London)* **102,** 319 (1943).
[317] W. O. Russell, E. T. Rouse, and J. A. Read, *Arch. Pathol.* **38,** 40 (1944).
[318] H. R. Perkins and S. S. Zilva, *Biochem. J.* **47,** 306 (1950).
[319] H. Bunting and F. R. White, *Arch. Pathol.* **49,** 590 (1950).
[320] S. M. Horvath and D. Tebbe, *J. Biol. Chem.* **165,** 657 (1946).

rapidly during the rest of the experimental period. Total phosphorus was lowered only slightly, but the acid-soluble phosphates, especially phosphocreatine, were lowered markedly. A reduction was also observed in the content of glycogen, total nitrogen, creatine, and muscle solids. When ascorbic acid was administered to the scorbutic animals, the phosphocreatine values became nearly normal. The opinion was expressed that the low levels of phosphocreatine and the interference with its synthesis may account for the general weakness and quick fatigability of the scorbutic animals.

Sadhu[89] found that the phospholipid content of the tissues was not altered in scurvy. The relation of ascorbic acid to the oxidation of unsaturated fatty acids in phospholipins was discussed in the section on fats. In certain tissues of scorbutic animals this oxidation is much depressed.[286]

(3) *Sodium and Potassium.* Randoin and Michaux[151] found the disturbances in sodium and potassium metabolism in scurvy to be relatively slight.

(4) *Iron.* Most of the reports of an influence of ascorbic acid on the iron content of tissues have been based on studies with human subjects. The more recent investigations tend to indicate that no special relationship exists. Schulze and Morgan[321] studied ascorbic acid with respect to its effectiveness in iron therapy in two matched groups of children (non-scorbutic and with less than normal, but not pathologic, hematological values) 7 to 12 years of age and living under identical conditions. Members of both groups were given 4 mg. of copper sulfate and 0.1 g. of iron as soluble ferric pyrophosphate daily. Members of one group received in addition 100 mg. of ascorbic acid daily. After treatment for 10 weeks it was found that the hemoglobin values were increased equally in the two groups. The hematocrit values and erythrocyte counts were only slightly affected. The response to iron therapy thus was not influenced by the administration of ascorbic acid. Totterman[322] conducted a study on the relationship between vitamin C and iron under normal physiological conditions and in infectious diseases with special reference to the pathogenesis of infectious anemia and found no connection between disturbances in vitamin C and iron in either condition. Lu[323] investigated the influence of L-ascorbic acid on iron storage in mice and found no significant difference in the iron content of the liver and spleen with and without the administration of ascorbic acid.

(5) *Manganese.* It has been suggested that the low level of manganese in the tissues of human beings or guinea pigs[324, 325] is related to their inabil-

[321] H. V. Schulze and A. F. Morgan, *Am. J. Diseases Children* **71**, 593 (1946).
[322] L. E. Totterman, *Acta. Med. Scand.* 134 *Suppl.* **134**, CCXXX (1949).
[323] C. C. Lu, *Chinese J. Physiol.* **17**, 173 (1949).
[324] C. C. Lund, L. A. Shaw, and C. K. Drinker, *J. Exptl. Med.* **33**, 231 (1921).

ity to synthesize vitamin C.[326] Manganese, however, is not involved in the synthesis of ascorbic acid in either the rat[327] or the guinea pig.[328] The injection of manganese into scorbutic guinea pigs resulted in no change in blood phosphatase levels or in concentration of indophenol-reducing substances in the liver.

(6) *Copper.* Lindow *et al.*[329] stated that copper is present in all biological fluids in a concentration sufficient to produce a rapid oxidation of the vitamin C. It is known, however, that copper does not oxidize all the vitamin C, owing to various protective mechanisms. De Caro[330] reported that excessive amounts of copper induce a higher demand for ascorbic acid in guinea pigs. However, Lesne *et al.*[331] investigated the role of copper in the utilization of ascorbic acid by the guinea pig and reported that addition of copper to a scorbutigenic diet prevented the development of symptoms of scurvy in the bones and joints and also caused these symptoms to recede if supplementation with copper was not begun until after the symptoms appeared. The animals receiving copper all lost weight, however, and none of them survived beyond the fifty-sixth day. These results are of considerable interest, but confirmatory evidence has not been reported. Hochberg *et al.*[332] conducted tests with human subjects to determine the effect of copper ingestion (7 mg.) on the fate of dietary ascorbic acid (200 mg. daily) and found no greater destruction as a result of the presence of the copper. The effect of prolonged ingestion of copper on the excretion of the vitamin was not determined. It is possible that under the conditions of these tests the reserves of copper in the body were sufficient to supply the small amounts needed for the catalytic oxidation of ascorbic acid since in the presence of ascorbic acid there tends to be a continuous regeneration of the catalyst. Amounts of copper in excess of this requirement would, therefore, not be expected to have noticeable effects on the rate of oxidation of the vitamin.

e. Effect on Other Vitamins

(1) *Vitamin A.* (a) Effect of Ascorbic Acid on the Level and Function of Vitamin A. Kimble and Gordon[333] in studies with human subjects observed that administration of ascorbic acid caused an increase in the blood

[325] W. F. Von Oettingen, *Physiol. Revs.* **15**, 175 (1935).
[326] M. N. Rudra, *Nature* **144**, 868 (1939).
[327] P. D. Boyer, J. H. Shaw, and P. H. Phillips, *J. Biol. Chem.* **143**, 417 (1942).
[328] J. T. Skinner and J. S. McHargue, *Am. J. Physiol.* **145**, 566 (1946).
[329] C. W. Lindow, C. A. Elvehjem, and W. H. Peterson, *J. Biol. Chem.* **82**, 465 (1929).
[330] L. De Caro, *Boll. soc. ital. biol. sper.* **13**, 727 (1938).
[331] E. Lesne, M. Polonovski, and S. Briskas, *Compt. rend.* **217**, 406 (1943).
[332] M. Hochberg, D. Melnick, and B. L. Oser, *J. Nutrition* **30**, 225 (1945).
[333] M. Kimble and E. S. Gordon, *J. Biol. Chem.* **128**, lii (1939).

levels of vitamin A, although there had been no increase in the intake of vitamin A. Bassett *et al.*[334] found that administration of ascorbic acid increased the plasma and tissue levels of vitamin A in foxes and mink and concluded that synthesis of vitamin C is dependent upon the supply of vitamin A. Mitolo[335] reported that the α- and β-carotene contents of the livers of scorbutic guinea pigs were somewhat lower than in normal animals. Significance of these results may be questioned because food intake was not controlled. Stewart[336] reported that ascorbic acid in daily doses of 150 mg. produced as great an improvement in dark adaptation of human subjects as did daily doses of 24,000 I.U. of vitamin A. The general trend of evidence appears to indicate that the vitamin C status may influence the blood and tissue levels of vitamin A.

(b) Effect of Vitamin A Level on Content of Ascorbic Acid—Deficiency or Inadequate Supply of Vitamin A. Several investigations have shown a reduction in the ascorbic acid content of the organs of vitamin A-deficient rats, but unfortunately in some of them there was no control of food intake.[335, 337-339] A similar effect has been shown in cattle,[340] but it was not found in mature chickens.[341] In studies of avitaminosis A in rats, Sjöberg[342] found that histological changes in the teeth and muscles paralleled the decreasing blood ascorbic acid level. He concluded that the organs which synthesize vitamin C are damaged in vitamin A deficiency. Meyer and Krehl[343] observed symptoms resembling scurvy in rats maintained on a vitamin A-deficient diet supplemented with B vitamins in crystalline form. The scurvy-like condition was associated with enlarged adrenals and a diminished ascorbic acid content of the liver, adrenals, and blood. The scorbutic symptoms but not the vitamin A-deficiency symptoms were curable with ascorbic acid. Guerrant[344a] conducted tests with rats using the U.S.P. vitamin A-deficient diet in which the B vitamins were supplied by brewer's yeast and found that the growth and physical condition of the groups receiving and not receiving ascorbic acid were similar, no scurvy-like symptoms being observed. He suggested that the variation in the form of

[334] C. F. Bassett, J. K. Loosli, and F. Wilke, *J. Nutrition* **35**, 629 (1948).
[335] C. Mitolo, *Boll. soc. ital. biol. sper.* **17**, 310 (1942).
[336] C. P. Stewart, *J. Physiol.* **96** *Proc.* 28 (1939).
[337] B. Sure, R. M. Theis, and R. T. Harrelson, *J. Biol. Chem.* **129**, 245 (1939).
[338] G. Jonsson, A. L. Obel, and K. Sjöberg, *Z. Vitaminforsch.* **12**, 300 (1942).
[339] M. Sangiorgi, *Boll. soc. ital. biol. sper.* **17**, 369 (1942).
[340] P. D. Boyer, P. H. Phillips, W. D. Pounden, C. W. Jensen, I. W. Rupel, and M. E. Nesbit, *J. Nutrition* **23**, 525 (1942).
[341] M. Rubin and H. R. Bird, *Poultry Sci.* **22**, 53 (1942).
[342] K. Sjöberg, *Kgl. Lantbruksakad. Tidskr.* **80**, 445 (1941).
[343] J. Meyer, and W. A. Krehl, *Arch. Biochem.* **16**, 313 (1948).
[344a] N. B. Guerrant, *Science* **108**, 724 (1948).

B vitamin supplements may have been responsible for the differences between these results and those of Meyer and Krehl.[343] The highly purified diet used by Meyer and Krehl may have been lacking in substances which act as stimulators of ascorbic acid synthesis.[344b] It also seems quite possible that the yeast may have equalized the food intake of the two groups and thus may have contributed to an equalization of ascorbic acid synthesis. That the synthesis of vitamin C may be related in some measure to food intake has been shown by Mapson and Walker.[345] They reported that in male rats deficient in vitamin A the lowered urinary excretion of ascorbic acid and its concentration in the liver and blood could be attributed to the lowered food intake, but in the females the concentration of ascorbic acid in both the liver and blood appeared to be unrelated either to food intake or to vitamin A deficiency. They also observed that the rise in urinary excretion of vitamin C in response to chloretone was less in vitamin A-deficient rats of both sexes than in the control animals, but the difference was eliminated if, in the control group, food intake was restricted to that of the deficient animals.

(*c*) Effects of Excess Vitamin A on Development of Scurvy. Mouriquand and Michel[346] reported that an excess of cod liver oil interferes with the cure of scurvy in guinea pigs. Collet and Eriksen[347] confirmed this finding and observed also that a moderate excess of vitamin A did not interfere with ascorbic acid. Tul'chinskaya[348] in a study of antagonism and synergism among vitamins observed that carotene lessens the capacity of guinea pig tissues to retain ascorbic acid. One milligram of carotene in 0.2 ml. of vegetable oil partially neutralized the effect of 5 mg. of ascorbic acid but appeared to have no effect against doses of 20 to 50 mg. of vitamin C. Oral administration of ascorbic acid was less effective than parenteral in increasing the tissue storage. Vedder and Rosenberg[349] reported that rats given large doses of jewfish oil with a high content of vitamin A developed scurvy-like symptoms and the ascorbic acid excretion was greatly reduced. Supplementation with 5 mg. of ascorbic acid per day gave almost complete protection. Rodahl[350] found that prolonged administration of excessive doses of vitamin A in the form of polar bear liver resulted in a scurvy-like condition in rats which could be alleviated with supplements of ascorbic acid, although it appeared certain that lack of ascorbic acid is not the only

[344b] H. E. Longenecker, R. R. Musulin, R. H. Tully, and C. G. King, *J. Biol. Chem.* **129**, 445 (1939).

[345] L. W. Mapson and S. E. Walker, *Brit. J. Nutrition* **2**, 1 (1948).

[346] G. Mouriquand and P. Michel, *Compt. rend. soc. biol.* Part 1, 1170 (1922).

[347] E. Collett and B. Eriksen, *Biochem. J.* **32**, 2299 (1938).

[348] K. Z. Tul'chinskaya, *Proc. Sci. Inst. Vitamin Research* (*U.S.S.R*) **3**, 218 (1941).

[349] E. B. Vedder and C. Rosenberg, *J. Nutrition* **16**, 57 (1938).

[350] K. Rodahl, *Nature* **164**, 530, 531 (1949).

factor in producing hypervitaminosis A. In further studies Rodahl[351] found that large doses of ascorbic acid did not afford significant protection against the effects of massive doses of vitamin A although supplying additional vitamin C was beneficial in counteracting a moderate excess of vitamin A. He stated, moreover, that the mechanism of the toxic effect of excess vitamin A on the cells remains obscure. Morehouse *et al.*[352] found that both the amount and concentration of ascorbic acid in livers of rats were affected by hypervitaminosis A. The concentration of ascorbic acid became progressively lower as the daily dosage of vitamin A was increased (20,000 to 80,000 units). Eeg-Larsen and Pihl[353] reported that the fall in serum ascorbic acid in rats treated daily with 20,000 to 40,000 units of vitamin A was the same as that in rats showing the same weight loss after paired feeding. Administration of additional ascorbic acid did not protect rats against excessive doses of vitamin A. It would appear from these results that control of food intake may be as important in the study of hypervitaminosis A as it has been shown to be in hypovitaminosis A.

(2) *Vitamin D.* Weld[354] reported that in human subjects vitamin D was more effective under some conditions than ascorbic acid in increasing capillary resistance. The possibility that vitamin D affects calcium as a component of the intercellular cement of the capillaries was not discussed. Bruce and Phillips [355] found no detectable effect of ascorbic acid upon the response to a small dose of vitamin D as determined by the "line test" using rats as experimental subjects. A possible effect of much larger doses of ascorbic acid than were employed would also have been of interest. Kyrki[356] found that administration of vitamin D caused no lowering of the ascorbic acid content of the blood serum in rachitic infants. Lecoq *et al.*[357a] fed the Randoin rachitogenic diet to young rats kept in the dark and found that large doses of ascorbic acid had no effect on the bone structure but prevented alkalosis and neuromuscular disturbances. Later[357b] these same investigators reported that calciferol appeared to exert a depressing effect on the storage of ascorbic acid in guinea pig tissues.

(3) *Vitamin K.* No direct relation between ascorbic acid and vitamin K has been shown, but lack of either or both substances reduces the prothrombin content of the blood.[147]

[351] K. Rodahl, Hypervitaminosis A. *Norsk Polarinstitutt, Oslo, Skrifter* **95**, (1950).
[352] A. L. Morehouse, N. B. Guerrant, and R. A. Dutcher, *Arch. Biochem. and Biophys.* **35**, 335 (1952).
[353] N. Eeg-Larsen and A. Pihl, *Acta Pharmacol. Toxicol.* **7**, 367 (1951).
[354] C. B. Weld, *J. Pediat.* **9**, 226 (1936).
[355] H. M. Bruce and G. E. Phillips, *Biochem. J.* **32**, 1 (1938).
[356] R. Kyrki, *Acta Pediat.* **31**, 428 (1943).
[357a] R. Lecoq, P. Chauchard, and H. Mazoué, *Compt. rend. soc. biol.* **138**, 835 (1944).
[357b] R. Lecoq, P. Chauchard, and H. Mazoué, *Compt. rend. soc. biol.* **220**, 631 (1945).

(4) *Vitamin E.* A number of substances, so-called antioxidants, may be associated with each other as synergists in protecting other substances against oxidation. Vitamin E is one of the most potent members of this group.

Golumbic and Mattill[358] observed that ascorbic acid increases the activity of tocopherols in preventing the oxidation of vegetable fats. Golumbic[359] found that ascorbic acid retarded the oxidation of tocopherol also in animal fat. Ascorbic acid was oxidized by the fat but at a much slower rate than was the tocopherol. Calkins and Mattill[360] had shown earlier that the oxidation of ascorbic acid is catalyzed by tocopherol in the presence of quinone esters of lard. Golumbic[359] suggested as an explanation of these phenomena that the antioxidant (tocopherol) was being continually regenerated at the expense of the synergist (e.g., ascorbic acid). Zacharias *et al.*[361] also found that ascorbic acid protects tocopherols against oxidation. Harris *et al.*[362] studied the content of vitamin C in guinea pigs on low ascorbic acid intake in association with variable vitamin E intake. Animals after receiving 6 to 10 mg. of ascorbic acid per kilogram of body weight and 0.5 to 1.0 mg. of supplementary tocopherol had a 19 to 22 % increase in ascorbic acid in the liver and a 22 to 66 % increase in the adrenals as compared to animals not receiving tocopherol. Dam *et al.*[363] found that the addition of 0.5 % ascorbic acid to a diet deficient in vitamin E prevented the development of symptoms of vitamin E deficiency in chicks. Storage of tocopherol in the fatty tissue was much greater in the chicks which received the ascorbic acid. The results suggest that ascorbic acid protects the tocopherol in the tissues. Farmer *et al.*[364] presented data which suggest a similar effect of ascorbic acid on vitamin E in guinea pigs. Fewer animals receiving 2 to 8 mg. of ascorbic acid per day, but no supplement of tocopherol, showed muscle degeneration than did those in the group which received only 1 mg. of vitamin C per day.

(5) *Thiamine.* Sure *et al.*[337] found that the ascorbic acid content of some of the organs of thiamine-deficient rats was notably reduced, whereas fasting for 10 to 11 days produced no change in concentration of the vitamin. Kasahara *et al.*[366] reported a growth-promoting effect of ascorbic acid

[358] C. Golumbic and H. A. Mattill, *J. Am. Chem. Soc.* **63**, 1279 (1941).
[359] C. Golumbic, *Trans. 1st Conf. Biol. Antioxidants, New York*, p. 42 (1946).
[360] V. P. Calkins and H. A. Mattill, *J. Am. Chem. Soc.* **66**, 239 (1944).
[361] L. Zacharias, P. Goldhaber, and V. E. Kinsey, *J. Nutrition* **42**, 359 (1950).
[362] P. L. Harris, K. C. D. Hickman, and M. R. Woodside, Meeting of the American Chemical Society, Buffalo, N. Y., September, 1942.
[363] H. Dam, I. Kruse, I. Prange, and E. Sondergaard, *Biochem. et Biophys. Acta* **2**, 501 (1948).
[364] F. A. Farmer, B. C. Mutch, J. M. Bell, L. D. Woolsey, and E. W. Crampton, *J. Nutrition* **42**, 309 (1950).
[366] M. Kasahara, Y. Nisigawa, and S. Hirao, *Klin. Wochschr.* **18**, 246 (1939).

when used as a supplement in addition to thiamine in rats maintained on diets deficient in vitamin B_1. They also observed a synergism of action between the two vitamins so marked as to reach an almost complete substitution, particularly of thiamine, by ascorbic acid. Aloisi and Polanyi[367] confirmed the synergism described by Kasahara *et al.* and reported success with ascorbic acid in curing within a few minutes rats affected with polyneuritis caused by thiamine deficiency. A possible important interrelation of ascorbic acid to pyruvate metabolism has been suggested in results obtained by Cavallini.[85a] A stimulating effect of vitamin C on pyruvate oxidation may account for the sparing action on thiamine. Zucker *et al.*[368] stated that thiamine increases the fixation of ascorbic acid by guinea pig tissues. Highet and West[369] studied the toxic effects produced by the administration of large doses of thiamine to scorbutic guinea pigs. They found that muscular weakness and emaciation were the most outstanding effects produced.

(6) *Riboflavin.* Sure *et al.*[337] observed a reduction in content of ascorbic acid in the tissues of riboflavin-deficient as compared to normal rats. Randoin and Raffy[370] found a 20 to 40% decrease in the riboflavin content of the kidneys and adrenals in scorbutic guinea pigs as compared to those of normal animals. The other organs showed no significant change. Unfortunately the animals were not pair fed. Ekman and Strömbeck[371] reported that rats on riboflavin-deficient diets live longer if supplied with ascorbic acid and suggested that the vitamin exerts its beneficial action by the oxidative detoxification of aromatic metabolic substances produced in the body.

(7) *Pantothenic Acid.* The interrelations of pantothenic acid and ascorbic acid with adrenal structure and function have been reviewed recently.[372] The excretion of these two vitamins was studied by Dumm and Ralli[373] in adrenalectomized and intact rats maintained on diets deficient in and supplemented with calcium pantothenate. Consideration of their data led the authors to suggest that the adrenal gland is concerned in ascorbic acid synthesis.

Daft[374] has observed a somewhat unexpected effect of ascorbic acid on pantothenic acid-deficient rats. Incorporation of 2% ascorbic acid in the pantothenic acid-free diet prevented development of all pantothenic acid

[367] M. Aloisi and V. Polanyi, *Boll. soc. ital. biol. sper.* **14**, 637 (1939); **15**, 451 (1940).
[368] L. Zucker, L. Hall, M. Young, and T. F. Zucker, *Growth* **5**, 399 (1942).
[369] D. M. Highet and E. S. West, *Proc. Soc. Exptl. Biol. Med.* **63**, 482 (1946).
[370] L. Randoin and A. Raffy, *Compt. rend. soc. biol.* **127**, 590 (1938).
[371] B. Ekman and J. P. Strombeck, *Acta Physiol. Scand.* **18**, 99 (1949).
[372] *Nutrition Revs.* **8**, 25 (1950).
[373] M. E. Dumm and E. P. Ralli, *Endocrinology* **45**, 188 (1949).
[374] F. B. Daft, *Federation Proc.* **10**, 380 (1951).

deficiency symptoms and permitted normal growth and development. More recent results from the same laboratory[375] suggest that this is a nonspecific effect.

(8) *Pteroylglutamic Acid.* Silverman and Mackler[376] found that pteroylglutamic acid (PGA) has no antiscorbutic effect in guinea pigs. Lepp *et al.*[377] reported that administration of ascorbic acid results in a small increase in hemoglobin in chicks when used with a diet partially deficient in PGA. Johnson and Dana[378] observed that supplying ascorbic acid to rats depleted of PGA produced a gain in weight and a return of the white cell count to normal and prevented further development of "chromodacryorrhea" (achroiocythemia?). The hemoglobin continued to decrease, however, and there was no reticulocyte response until after administration of PGA. Woodruff and Darby[252] also found a relation between ascorbic acid and PGA. The administration of either PGA or ascorbic acid to guinea pigs on a scorbutigenic diet containing 5% tyrosine caused a decreased excretion of tyrosyl derivatives and keto acids. Dietrich *et al.*[379] observed that ascorbic acid stimulates the synthesis of folic acid in the chick and considered the stimulatory action to be that of an oxidation-reduction mechanism rather than one of a specific vitamin action. Recent investigations[380, 381] have shown that folic acid is present in natural materials in the form of folinic acid, and it has been demonstrated that ascorbic acid enhances the conversion of folic to folinic acid both *in vivo*[382] and *in vitro*.[383] The suggestion has been advanced that the change from folic to folinic acid is associated with the transfer of single carbon units. It has also been postulated that it is not only the complete folinic acid molecule as such which has biological activity, but also tetrahydrofolic acid.[384] The exact role of ascorbic acid in the folic-folinic acid relation is not known, but it is possible that it has a stabilizing effect on folinic acid, owing to its antioxidant action. The finding of

[375] J. M. Hundley and R. B. Ing, *Federation Proc.* **12**, 417 (1953).

[376] F. N. Silverman and B. Mackler, *Proc. Soc. Exptl. Biol. Med.* **76**, 574 (1951).

[377] A. Lepp, P. R. Moore, C. A. Elvehjem, and E. B. Hart, *Poultry Sci.* **26**, 594 (1947).

[378] B. C. Johnson and A. S. Dana, *Science* **108**, 210 (1948).

[379] L. S. Dietrich, C. A. Nichol, W. J. Monson, and C. A. Elvehjem, *J. Biol. Chem.* **181**, 915 (1949).

[380] J. A. Brockman, Jr., B. Roth, H. P. Broquist, M. E. Hultquist, J. M. Smith, M. J. Fahrenbach, D. B. Cosulich, R. P. Parker, E. L. R. Stokstad, and T. H. Jukes, *J. Am. Chem. Soc.* **72**, 4325 (1950).

[381] K. Schwarz, *Federation Proc.* **10**, 394 (1951).

[382] C. A. Nichol and A. D. Welch, *Proc. Soc. Exptl. Biol. Med.* **74**, 52 (1950).

[383] W. Shive, T. J. Bardos, T. J. Bond, and L. L. Rogers, *J. Am. Chem. Soc.* **72**, 2817 (1950).

[384] H. P. Broquist, M. J. Fahrenbach, J. A. Brockman, Jr., E. L. R. Stokstad, and T. H. Jukes, *J. Am. Chem. Soc.* **73**, 3535 (1951).

May and coworkers[385] that megaloblastic anemia in monkeys, associated with a deficiency of ascorbic acid, could be cured promptly either by very small amounts of folinic acid or ascorbic acid suggests such a relationship.

(9) *Vitamin B_{12}.* Dietrich *et al.*[379] found that addition of both ascorbic acid and vitamin B_{12} to chicks on a semipurified diet resulted in greater growth than with either one alone. As previously mentioned, ascorbic acid has been shown to have a growth-promoting effect on *L. leichmannii*, an organism which requires vitamin B_{12} also. On the other hand, reports have been made of a destructive action of ascorbic acid on vitamin B_{12} in solution. Gakenheimer and Feller[386] found that more than 50 % decomposition of vitamin B_{12} occurred in solutions containing ascorbic acid within 24 hours when stored at 28 to 30°. Lang and Chow[387] also observed that ascorbic acid caused a destruction of vitamin B_{12} on standing, the rate of change varying with pH.

(10) *Summary of Vitamin Interrelations.* (a) Effect of Ascorbic Acid on Other Vitamins. Ascorbic acid appears to influence the blood and tissue levels of vitamin A. It protects tocopherol against oxidation in the tissues, has a sparing or functional effect on members of the B complex, and may possibly influence the synthesis of some of them.

(b) Effect of Other Vitamins on Ascorbic Acid. Vitamin A appears to have a stimulatory effect on the synthesis of vitamin C in the rat; in excessive amounts it may lessen the capacity of the tissues to retain vitamin C. Vitamin E has a protective effect on ascorbic acid. Thiamine, riboflavin, and pantothenic acid have a stimulatory effect on ascorbic acid synthesis in rats, and thiamine and riboflavin may affect fixation of the vitamin in the tissues of guinea pigs.

5. Enzymes Affected by Ascorbic Acid Deficiency

Ascorbic acid has marked effects on some types of enzymes. The systems which showed a marked drop in activity in scurvy are succinic dehydrogenase,[388] alkaline phosphatase in bones[315, 389] and skin, phosphorylase, and the enzyme controlling tyrosine oxidation.[207b, c] Enzymes which have been reported to become overactive in the deficiency are adenosine triphos-

[385] C. D. May, E. N. Nelson, R. J. Salmon, C. U. Lowe, R. I. Lienke, and D. Sundberg, *Bull. Univ. Minn. Hosp.* **21**, 208 (1950).
[386] W. C. Gakenheimer and B. A. Feller, *J. Am. Pharm. Assoc. Sci. Ed.* **38**, 660 (1949).
[387] C. A. Lang and B. F. Chow, *Federation Proc.* **9**, 193 (1950).
[388] C. J. Harrer and C. G. King, *J. Biol. Chem.* **138**, 111 (1941).
[389] G. H. Bourne, *Quart. J. Exptl. Physiol.* **32**, 1 (1943).

TABLE X

CHANGES IN ENZYME ACTION IN ASCORBIC ACID DEFICIENCY IN GUINEA PIGS

Enzyme	Source and nature of test material	Reference	Activity and/or amount of enzyme
Succinic dehydrogenase	Homogenized heart muscle-suspension; skeletal muscle extract	Harrer and King[388]	Slight decrease in heart; marked decrease in skeletal muscle
Cytochrome oxidase	Extracts of liver	Phillips, Stare, and Elvehjem[393]	Markedly diminished
Cytochrome oxidase	Skeletal muscle, heart, and brain extracts	Harrer and King[388]	Decreased activity in heart and skeletal muscle
Cytochrome oxidase	Extracts of organs	D'Agata[394]	Substantial decrease
Phosphorylase	Extracts of liver and kidney	Murray[390]	Marked decrease in ability of liver to phosphorylate substrates; difference less in kidney tissue
Enzyme (?) for oxidation of tyrosine	Kidney and liver slices	Lan and Sealock[395]	Scorbutic liver slices do not show increased oxygen consumption in presence of tyrosine
Enzyme (?) for oxidation of tyrosine	Liver suspensions	Painter and Zilva[249]	Rate of disappearance of phenolic group of L-tyrosine retarded
Enzyme (?) for oxidation of tyrosine	Liver slices, homogenates, and supernatant	Rienits[250a]	Slices—activity reduced; homogenates—no difference; supernatant—reduced
Alkaline phosphatase	Serum	Todhunter and Brewer[298]	Increased slightly in mild scurvy; decreased markedly in severe scurvy
Alkaline phosphatase	Serum	Scoz, Cattaneo, and Gabbrielli[402]	Increase in early stages followed by decrease
Alkaline phosphatase	(a) Intestinal mucosa, kidney, adrenal, and liver; (b) bone and serum	Gould and Schwachman[315]	No change in (a); striking decrease in (b)
Alkaline phosphatase	Histochemical tests on bone	Bourne[389]	Marked decrease
Alkaline phosphatase	Histochemical tests on kidney	Russell, Rouse, and Read[317]	Moderate decrease
Alkaline phosphatase	Histochemical tests on healing skin wounds	Danielli, Fell, and Kodicek[55]	There appears to be some relation between enzyme activity and the amount of vitamin C available
Alkaline phosphatase	Serum and extracts of liver, intestine, kidney, and bone	Perkins and Zilva[318]	No change in early stages; activities of all decreased in late stages
Alkaline phosphatase	Histochemical study of skin wounds	Bunting and White[319]	Greatly reduced
Alkaline phosphatase	Filtrate from suspensions of intestinal mucosa, kidney cortex, brain and liver	Harrer and King[388]	Not much change
Glucuronidase	Liver	Becker and Friedenwald[403]	Activity of enzyme reduced by as much as 90%
Aldolase	Extracts of liver, kidney, and muscle	Murray[390]	No difference
Cathepsin	Effect of cathepsin on autolysis of liver	Okuda[397]	Autolysis sharply depressed
Catalase	Blood	Török and Neufeld[398] Tria[399]	Decrease in catalase concentration in 7 to 10 days on scurvy diet
Esterase	Blood	Palladin and Normark[392]	Amount greatly decreased

TABLE X—*Continued*

Enzyme	Source and nature of test material	Reference	Activity and/or amount of enzyme
Esterase	Liver	Pantschenko-Jurewicz and Kraut[400]	Considerably decreased
Esterase	Fresh liver suspensions and extracts from acetone-dried liver	Harrer and King[258]	Decreased progressively with vitamin depletion to −65%
Lipase	Hepatic duodenal	Bori and Cera[401]	Marked decrease
Catalase	Blood	Palladin[392] and Normark	No definite change
Protease	Liver	Martinson[391]	Increase
	Blood	Palladin[392]	Increase
Adenosinetriphosphatase	Extracts of liver	Murray[390]	Increase

phatase[390] and proteolytic enzymes of liver[391] and blood.[392] Table X summarizes contributions made in studies with enzymes.

6. Respiration

There is considerable evidence that ascorbic acid exerts an influence on cellular respiration, but little is actually known as to the mechanisms by which the effects are produced. A role as a reducing agent has frequently been postulated. King[404] suggested, however, that rather than functioning as a major hydrogen carrier itself ascorbic acid may serve as a regulating and protective agent, possibly for other hydrogen carriers and other enzyme systems. As previously mentioned, much evidence has accumulated which shows that the vitamin can stimulate oxidation, particularly that of certain types of amino acids, fatty acids, phospholipids, and pyruvate.

Definite progress has been made in partially elucidating a role of ascorbic acid in respiration by the discovery of its function in the oxidation of the side chain of tyrosine.[247, 396, 405] As previously mentioned, Sealock and Good-

[390] H. C. Murray, *Proc. Soc. Exptl. Biol. Med.* **75**, 598 (1950).
[391] E. E. Martinson, I. V. Fetsenko, L. Sokolova, V. Solanikova, and Y. Troitskii, *Bull. soc. chim. biol.* **19**, 1521 (1937).
[392] A. Palladin and P. Normark, *Biochem. Z.* **152**, 420 (1924).
[393] P. H. Phillips, F. J. Stare, and C. A. Elvehjem, *J. Biol. Chem.* **106**, 41 (1934).
[394] A. D'Agata, *Atti accad. peloritana, Classe sci. fis. mat. e. nat.* **49**, 224 (1947).
[395] T. H. Lan and R. R. Sealock, *J. Biol. Chem.* **155**, 483 (1944).
[396] R. R. Sealock and T. H. Lan, *J. Biol. Chem.* **167**, 689 (1947).
[397] H. Okuda, *Sei-i-kai Med. J.* **57**, 4 (1938).
[398] G. Torök and L. Neufeld, *Klin. Wochschr.* **13**, 1205 (1934).
[399] E. Tria, *Atti reale accad. Italia, Mem. classe sci. fis. mat. e. nat.* **3**, 806 (1942).
[400] W. von Pantshenko-Jurewicz and H. Kraut, *Biochem. Z.* **285**, 407 (1936).
[401] D. V. Bori and B. Cera, *Quaderni nutriz.* **6**, 301 (1939).
[402] G. Scoz, C. Cattaneo, and M. C. Gabbrielli, *Enzymologia* **3**, 29 (1937).
[403] B. Becker and J. S. Friedenwald, *Arch. Biochem.* **22**, 101 (1949).
[404] C. G. King, *Cold Spring Harbor Symposia Quant. Biol.* **7**, 137 (1939).
[405] W. J. Darby, R. H. DeMeio, M. L. C. Bernheim, and F. Bernheim, *J. Biol. Chem.* **158**, 67 (1945).

land[207b] have presented evidence suggesting that "ascorbic acid plays its part in tyrosine metabolism by acting as a coenzyme in the oxidation of the deaminated amino acid." They postulated that "the enzyme would be regarded as removing hydrogen from the tyrosine keto acid by means of the dehydroascorbic acid form of the vitamin, with subsequent transfer to oxygen and regeneration of dehydroascorbic acid." Such a regeneration of the dehydroascorbic acid would aid in maintaining a balance between the two forms of the vitamin in the catalytically induced oxidation-reduction cycle described by Barron *et al.*[84] in which hydrogen peroxide is presumably formed. The oxidative action of hydrogen peroxide in association with ascorbic acid in influencing polymerization of mucopolysaccharides and in promoting oxidation of unsaturated fatty acids has been discussed previously. Cavallini[85a] reported results which suggest that the physiological role of ascorbic acid may be to form a continuous supply of hydrogen peroxide. He found that oxidation of ascorbic acid with $CuCl_2$ in a Warburg apparatus resulted in oxidation of added pyruvate and the amount of pyruvate oxidized was proportional to the rate of oxidation of ascorbic acid and was independent of the catalyst employed. The oxidizing action on pyruvate was considered to be due to the production of hydrogen peroxide during the oxidation of ascorbic acid. He postulated that in the organism ascorbic acid is present in a state of dynamic balance with respect to oxidation and reduction, creating thereby a constant flow of hydrogen peroxide proportional to the amount of the vitamin and to the agents which reduce it, i.e., cytochrome c and sulfhydryl compounds. Cavallini considered it equally possible that coupled oxidation reactions can take place under defense conditions on the part of the disturbing substances or by a closely connecting mechanism due to which there is a direct transposition of elements in the molecular complex rather than a two-phase reaction comprising first a hydrogen peroxide formation and then an action of the latter on the substrate.

More information is needed before a full interpretation can be made of the role of ascorbic acid in cellular respiration. At present no definite explanation can be made of the increased respiratory rate which has been observed[85b, 404] to occur during the onset of scurvy, but it seems possible, as previously stated, that with a deficiency or lack of the vitamin the normal oxidation-reduction cycle is upset, resulting in imbalances probably involving the action of hormones, enzymes,[406] and other vitamins.

7. Excretion and Retention

The kidneys have a rather definite threshold for excretion of ascorbic acid. A rapid rise in excretion of the vitamin occurs in man when the plasma

[406] R. R. Sealock and R. L. Goodland, *Science* **114**, 645 (1951).

concentration exceeds a certain level which lies, according to different investigators, at 1 to 1.3 mg.%,[407] at 1.4 mg.%,[408] or at 1.5 mg.%.[409] In the guinea pig the threshold appears to lie[226] within the range reported for man. When a large dose of the vitamin was given intraperitoneally to guinea pigs accustomed to large daily doses, there was a marked elevation of the levels in the blood, liver, and kidneys but little change in those of the spleen and adrenals.[226, 230] Soon after its administration considerable urinary excretion of the vitamin occurred, particularly in adult animals. The amount taken up by the organs following injection depended on the degree of depletion since the last injection. During the period between the daily injections the unsaturation of the tissues becomes greater in young rapidly growing animals than in mature animals. Usually not more than one-fourth of the injected dose (5 mg. per 100 g.) was excreted in the urine by the full-grown animals, but only half as much per unit of body weight was excreted by the growing animals. Under the high dosage conditions of these experiments a considerable amount of the vitamin was excreted into the lumen of the gastrointestinal tract and apparently was there destroyed. As much as 15% of the total injected dose was to be found at one time in the contents of the stomach and small intestine but only very small amounts in the cecum and large intestine. Since in the guinea pig the contents of the stomach and small intestine are passed fairly rapidly into the cecum,[410] the results suggested that a destruction within the gastrointestinal tract of 15% and probably considerably more of the amount injected must have occurred.

Penny and Zilva[225] stated that gastrointestinal absorption of ascorbic acid is much more rapid and efficient in man than in the guinea pig. The cause for this difference may be attributable, at least in part, to the more rapid movement of the contents of the stomach and small intestine in the guinea pig which thereby allows insufficient time for absorption of the vitamin. Differences were found between the growing and mature animals with respect to the manner in which they handled the vitamin, the younger animals appearing to have a considerably greater capacity for its conservation. Some loss of ascorbic acid also is reported to occur in man in the feces and in sweat. Chinn and Farmer[411] reported a daily excretion in the feces of normal human individuals of approximately 5 mg. Martin[412] found slightly less. After catharsis or in acute diarrhea, there may be an increased

[407] A. M. Klosterman, J. E. Haines, H. Hauck, and A. B. Kline, *J. Nutrition* **33**, 505 (1947)

[408] J. M. Faulkner and F. H. L. Taylor, *J. Clin. Invest.* **17**, 69 (1938).

[409] G. J. Friedman, S. Sherry, and E. P. Ralli, *J. Clin. Invest.* **19**, 685 (1940).

[410] M. E. Reid and W. C. White, *Am. J. Physiol.* **152**, 455 (1948).

[411] H. Chinn and C. J. Farmer, *Proc. Soc. Exptl. Biol. Med.* **2**, 561 (1939).

[412] H. Martin, *Klin. Wochschr.* **20**, 287 (1941).

fecal excretion of the vitamin, probably owing to failure in absorption.[413] Before drawing conclusions, however, as to the presence of ascorbic acid in the feces, tests should be conducted to eliminate reductone and reductic acid as possible interfering substances.[414, 415] Losses in sweat are negligible unless sweating is very profuse or the intake of the vitamin low.[416-418]

The ability of the tissues to retain ascorbic acid depends somewhat on factors other than the degree of saturation of the tissues of the body. Included among these other factors are: (a) intake of acids and alkalies, (b) nature of the diet, (c) the vitamin as it occurs in foods as compared to the synthetic product, (d) effects of fever, (e) trauma, and (f) exercise.

a. Intake of Acids and Alkalies

Hawley *et al.*[419] made a study of the effect of pH changes of the urine resulting from ingestion of sodium bicarbonate and ammonium chloride in relation to urinary excretion of the vitamin in human subjects. They found a marked decrease in output when the urinary pH was in the alkaline range 7.5 to 8.1. The plasma ascorbic acid level was lowered by ingestion of either sodium bicarbonate or ammonium chloride. The lowered excretion of the vitamin which occurs after ingestion of sodium bicarbonate is probably a result of increased destruction during the excretion process, and the increased excretion of the vitamin after ammonium chloride ingestion may be a result of increased preservation during the excretory process. Hawthorne and Storvick[420] made a study with similar results indicating an interference with normal utilization.

b. Nature of the Diet

Little is known about this factor. Patterson and Bourquin[421] reported less than a 2% difference in ascorbic acid excretion in six of eight women subjects on a balanced diet high in protein as compared to the excretion on a normal diet. The amount of the vitamin necessary for saturation was slightly greater for the high protein diet. Similar studies on the effect of

[413] A. F. Abt, C. J. Farmer, and Y. J. Topper, *Proc. Soc. Exptl. Biol. Med.* **43**, 24 (1940).
[414] M. Miller, *Food Research* **12**, 343 (1947).
[415] E. M. Hewston, M. Fisher, and E. Orent-Keiles, *U. S. Dept. Agr. Tech. Bull.* **1023** (1951).
[416] D. M. Tennent and R. H. Silber, *J. Biol. Chem.* **148**, 359 (1943).
[417] O. Mickelsen and A. Keys, *J. Biol. Chem.* **149**, 479 (1943).
[418] J. B. Shields, B. C. Johnson, T. S. Hamilton, and H. H. Mitchell, *J. Biol. Chem.* **161**, 351 (1945).
[419] E. E. Hawley, R. G. Saags, and D. J. Stephens, *J. Nutrition* **14**, 1 (1937).
[420] B. E. Hawthorne and C. A. Storvick, *Proc. Soc. Exptl. Biol. Med.* **67**, 447 (1948).
[421] I. Patterson and A. Bourquin, *Am. J. Digest. Diseases* **10**, 390 (1943).

variations in the fat content of the diet are lacking but undoubtedly should be made.

c. Ascorbic Acid in Foods as Compared to the Synthetic Product

Several reports have indicated a greater retention or positive effect of the naturally occurring vitamin.[295, 422-431] Other workers, however, have reported finding equal biological potency of the naturally occurring and synthetic forms.[432-435] Melnick *et al.*[436a] investigated the problem using their human bioassay method.[436b] They found that when ascorbic acid was added to apple juice there was no increase in availability above that of the synthetic form but the vitamin in the natural juice was more stable than that in the aqueous solution. The naturally occurring vitamin is associated with soluble carbohydrates, organic acids other than ascorbic, flavonoids of certain types, other water-soluble vitamins, minerals, and also other types of substances. It seems possible that under some conditions one or more of these factors may be involved in producing effects either surpassing or less than those obtained with the synthetic vitamin. However, there is no indication that the synthetic product itself is less effective than the vitamin contained in natural foodstuffs.

d. Fever

Hausberger and Nevenschwander-Lemmer[437] found that injected ascorbic acid disappears from the blood of patients with fever more quickly than it does from normal persons. Stutskii[438] and Marotta and Calendoli[439]

[422] E. Jacobsen, *Skand. Arch. Physiol.* **72**, 259 (1935).
[423] A. Elmby and E. Warburg, *Lancet* **II**, 1363 (1937).
[424] E. N. Todhunter, R. C. Robbins, G. Ivey, and W. Brewer, *J. Nutrition* **19**, 113 (1940).
[425] G. Wilhelmi, *Z. ges. exptl. Med.* **107**, 551 (1940).
[426] S. K. Roy and M. N. Rudra, *Ann. Biochem. and Exptl. Med.* (*India*) **1**, 307 (1941).
[427] W. W. Spink, S. Agnew, and O. Mickelsen, *J. Immunol.* **44**, 289, 297 (1942).
[428] A. Kuhn and H. Gerhard, *Hippokrates* **12**, 1284 (1941); *Chem. Zentr.* **1**, 1155 (1942).
[429] Alter, *Z. Vitaminforsch.* **12**, 297 (1942).
[430] A. A. Selenzneva and N. V. Kukina, *Vitamin Research News* (*U.S.S.R.*) **1**, 40 (1946).
[431] E. W. Crampton and E. W. Burton, *Federation Proc.* **5**, 230 (1946).
[432] A. Scheunert and J. Reschke, *Vitamine u. Hormone* **1**, 195 (1941).
[433] J. Hangartner and T. Gordonoff, *Z. Vitaminforsch* **12**, 226 (1942).
[434] E. R. Hartzler, *J. Nutrition* **30**, 355 (1945).
[435] K. J. Elliott and C. Schuck, *J. Am. Dietet. Assoc.* **25**, 845 (1949).
[436a] D. Melnick, M. Hochberg, and B. L. Oser, *J. Nutrition* **34**, 409 (1947).
[436b] D. Melnick, M. Hochberg, and B. L. Oser, *J. Nutrition* **30**, 67 (1945).
[437] F. X. Hausberger and N. Nevenschwander-Lemmer, *Klin. Wochschr.* **18**, 1119 (1939).
[438] I. L. Stutskii, *Klin. Med.* (*U.S.S.R.*) **15**, 1084 (1937); *Chem. Zentr.* **1**, 4680 (1938).
[439] G. Marotta and G. Calendoli, *Boll. soc. ital. biol. sper.* **19**, 16 (1944).

observed a marked decrease in urinary excretion of ascorbic acid by typhus patients during the period of high fever. There is evidence also of decreased excretion of the vitamin by tuberculosis patients.[440-443] Increased utilization of ascorbic acid in artificially induced fevers in guinea pigs was reported by Dobbelstein.[444] The level in the blood dropped to half its normal value. The same was true when the pigs were overheated in an incubating chamber at 37.5°. The accelerated metabolism associated with the elevated temperature was considered the cause of the increased consumption of the vitamin. Other workers have also reported finding an increased need for ascorbic acid in artificial fevers.[445-447]

e. Trauma

Kramarov[448] investigated the excretion of ascorbic acid by surgical patients and found that subjects with bone traumas excreted less ascorbic acid than those with other types of injury, especially during the first few days after injury. By the tenth day the excretion level was stabilized and remained unchanged for a month. Andrae and Browne[449] also studied ascorbic acid excretion after trauma in human subjects and found that the injured patients had a low output even after large doses of the vitamin and that retention was more marked after burns than after fractures. Repeated assays showed that, after injury and with high intake of the vitamin, the level in the blood fell, suggesting either rapid utilization or destruction. The urinary excretion was related closely to the level in the blood. As a possible index of the content in the tissues, the ascorbic acid values of the leucocytes were determined. In normal subjects they varied from 6.7 to 16.9 mg. per 100 ml., and on the high intake rose to 30 to 40 mg. within 7 days; in the subjects with traumas the values were low and failed to rise above 20 mg. per 100 ml. after 15 to 20 days on the high intake. The retention was greatest immediately after injury and decreased later as new tissue increased in the healing process, a fact which was considered as suggesting the improbability of the vitamin being used directly in the formation of new tissues.

[440] F. H. Heise and G. J. Martin, *Proc. Soc. Exptl. Biol. Med.* **34**, 642 (1936).
[441] G. Scoz, *Boll. soc. ital. biol. sper.* **11**, 908 (1936).
[442] M. A. Abbasy, L. J. Harris, and P. Ellman, *Lancet* **II**, 181 (1937).
[443] E. H. J. Warns, *Ned. Tijdschr. Geneesk.* **82**, 4426 (1938).
[444] O. Dobbelstein, *Z. ges. exptl. Med.* **107**, 532 (1940).
[445] K. Daum, K. Boyd, and W. D. Paul, *Proc. Soc. Exptl. Biol. Med.* **40**, 129 (1939).
[446] Von Falke, *Klin. Wochschr.* **18**, 818 (1939).
[447] S. L. Osborne and C. J. Farmer, *Proc. Soc. Exptl. Biol. Med.* **49**, 575 (1942).
[448] I. A. Kramarov, *Khirurgiya* **11**, 27 (1940).
[449] W. A. Andrae and J. S. L. Browne, *Can. Med. Assoc. J.* **55**, 425 (1946).

8. Chemotherapeutic, Immunologic, and Allergic Relations

a. Bacterial Diseases

Ascorbic acid is often credited with assisting the body to resist infection, particularly with respect to those diseases which are associated with an elevation of body temperature. Among the diseases in which the requirement of the vitamin appears to be increased (on the basis of excretion studies) are tuberculosis, rheumatic fever, and pneumonia, the increased need undoubtedly being caused by the elevated body temperature. Bartlett *et al.*[450] stated that their mortality figures in human cases were highly suggestive and that complete saturation of the tissues with ascorbic acid aided in diminishing infection. Findlay[451] produced a prescorbutic condition in guinea pigs maintained on a diet of oats, bran, and autoclaved milk supplemented with 2 ml. of orange juice every third day, and determined their resistance to four species of pathogenic bacteria. In all cases the pigs on the lower ascorbic acid intake, although showing no obvious signs of scurvy, succumbed to smaller infecting doses than did the controls. Werkman *et al.*[452] also reported that resistance of guinea pigs to bacterial infection was diminished when they were kept on diets deficient in vitamin C. The reduced temperature, characteristic of the scorbutic state in guinea pigs, was considered of significance in reducing resistance. Schmidt-Weyland and Költzsch[453] found that guinea pigs on a scorbutigenic diet were much more susceptible than normal animals to infections induced by inhalation and/or feeding with a mixture of penumococci and a fowl cholera pasteurella strain. Many more deaths from penumonia occurred among the scorbutic animals. Perla and Mamorsten[454] reported that in guinea pigs receiving insufficient amounts of ascorbic acid a chronic infection of low virulence may develop more rapidly and severely than in normal animals.

If ascorbic acid does have a beneficial influence on the course of certain types of infection in animals, the effect may presumably result from (1) an increase in the formation of, or availability of, immune bodies, (2) detoxification of harmful products of bacterial action, (3) altered permeability of membranes, or (4) increased phagocytic activity.

(1) *Relation of Ascorbic Acid to Immune Bodies.* Several investigators have reported favorable effects of vitamin C on the production of anti-

[450] M. K. Bartlett, A. E. Ryan, and G. D. Drummey, *New Engl. J. Med.* **229**, 642 (1943).

[451] G. M. Findlay, *J. Pathol. Bacteriol.* **26**, 1 (1923).

[452] C. H. Werkman, V. E. Nelson, and E. I. Fulmer, *J. Infectious Diseases*, **34**, 447 (1924).

[453] P. Schmidt-Weyland and W. Költzsch, *Z. Hyg. Infektionskrankh.* **108**, 199 (1928).

[454] D. Perla and J. Mamorsten, *Arch. Pathol.* **23**, 683 (1937).

bodies.[455-458] However, Naccari[459] found no such effects of the vitamin. He studied the influence of ascorbic acid on the bactericidal potency of whole blood and found that, in rabbits supplied with 50 mg. of ascorbic acid daily for 6 days, the whole blood showed no changes in bacterial action against *Streptococci*, *Staphylococci*, *Diplococci*, *S. paratyphi B*, *E. typhi*, and *E. coli*. He concluded that the intrinsic bactericidal potency of the blood has little importance as an indicator of immunity. Spink *et al.*[427] studied the relation of ascorbic acid to human complement and found that neither *in vitro* nor *in vivo* addition of ascorbic acid to serum deficient in the vitamin produced any change in the complement titer. In another report[460] these investigators observed the effect of ascorbic acid upon the bactericidal action of human blood with the vitamin injected intravenously into ascorbic acid-deficient subjects. It did not cause an increase in the bactericidal action of whole blood for coagulase-negative *Staphylococci* or in the bactericidal titer of serum for *E. coli* and *E. typhosa*. Furthermore, they found that complete oxidation of ascorbic acid by copper did not decrease the bactericidal action of normal human serum for *E. coli*, *E. typhosa*, and *Shigella paradysenteriae Flexner*. Perla and Mamorsten[454] stated that it would appear that alterations in natural resistance in scurvy are probably independent of any interference in the production of immune antibodies or in the availability of so-called natural antibodies as opsonins, bacteriolysins, and complement. The usual practice has been to relate the immunological relations of the vitamin to its level in the plasma. Possibly a closer approach would be to relate the reactions to the higher and less fluctuating level in the leucocytes. A recent report by Long[461] suggests that a modification of the conclusion of Perla and Mamorsten may be necessary. He found a lower level of serum antitoxin in guinea pigs maintained on a diet very low in ascorbic acid as compared to normal controls. Three groups of fifteen animals each were used (350 g. wt.); one group received unlimited cabbage, another received 5 mg. ascorbic acid three times weekly, and the third group had no supplement. The animals all grew equally well with no signs of scurvy at autopsy. On the thirty-fifth day an injection of diphtheria toxoid was made. Again on the sixty-third day graded doses of diphtheria toxin were injected subcutaneously and the diameter of the inflammatory lesion measured after 24 hours. Only slight differences were seen in the three groups, but 7 days later an-

[455] J. H. Jusatz, *Z. Immunitätsforsch.* **88**, 472, 483 (1936).
[456] R. R. Madison and W. H. Manwaring, *Proc. Soc. Exptl. Biol. Med.* **37**, 402 (1937).
[457] T. Izutumi and I. Sawai, *Japan. Z. Microbiol. u. Pathol.* **33**, 175 (1939).
[458] E. E. Ecker, L. Pillemer, J. J. Griffitts, and W. P. Schwartz, *J. Am. Med. Assoc.* **112**, 1449 (1939).
[459] A. Naccari, *Boll. ist. sieroterap. milan.* **20**, 161 (1941); *Chem. Zentr.* **1**, 1394 (1942).
[460] W. W. Spink, S. Agnew, O. Mickelsen, and L. M. Dahl, *J. Immunol.* **44**, 303 (1942).
[461] D. A. Long, *Brit. J. Exptl. Pathol.* **31**, 183 (1950).

other dose of toxoid was given. After 10 days the antitoxin content of the serum was determined. The average titer of the unsupplemented animals was about $\frac{1}{30}$ that of the other two groups.

(2) *Detoxification of Harmful Products of Bacterial Action.* Unfortunately, little is known about possible detoxifying action of ascorbic acid on bacterial poisons. Conjugates of the vitamin and these substances have not been found.

(3) *Altered Permeability of Membranes.* It is possible that changes in resistance to infection in scorbutus may be in part attributable to altered permeability of membranes, especially in view of Meyer's[88] finding of widespread liquefaction of the cytoplasm and disappearance of the cell walls in scurvy. Gersh and Catchpole's[76] recent study showing a breakdown of the basement membrane in scurvy also tends to support this suggestion.

(4) *Relation of Ascorbic Acid to Phagocytic Activity.* The chief effect of ascorbic acid on bacterial infection is related to its stimulative influence on phagocytic activity. Meyer[88] observed as a rule a striking absence of active phagocytosis by polymorphonuclear cells in intensely hemorrhagic subcutaneous tissue in scorbutic guinea pigs. There was also little phagocytosis in areas of hemorrhage in the skeletal or visceral musculature. Lawrynowicz[462] observed a reduction in phagocytosis with respect to *B. coli* and *Myco. tuberculosis*. Injection intraperitoneally of irritating substances into scorbutic animals resulted in an exudate which was not as rich in leucocytes as that obtained in normal animals. Messina and Varga[463] showed that ascorbic acid increases phagocytosis *in vitro*. Hunt[53] found that deficiency of the vitamin delayed the removal of cat gut ligatures in experimental wounds of guinea pigs either by phagocytosis or by extrusion. Cottingham and Mills[464] observed that vitamin C deficiency affects phagocytosis of bacteria by leucocytes. Bourne[465] found by histochemical methods that leucocytes normally absorb large amounts of ascorbic acid, particularly when they migrate into an injured or infected area. Meyer and Meyer[52] studied the pathology of *Staphylococcus* abscesses in vitamin C-deficient guinea pigs. In the scorbutic animals the appearance of macrophages was delayed and the number was less than normal. Polymorphonuclear reaction was prompt, but after a few days phagocytosis was less than normal. The investigation of Nungester and Ames[21] has furnished the most convincing demonstration of the influence of ascorbic acid on phagocytic activity. They made a study of the relationship between the ascorbic acid content of polymorphonuclear leucocytes in peritoneal exudates and the phagocytic

[462] A. Lawrynowicz, *J. physiol. et pathol. gén.* **29**, 270 (1931).
[463] A. Messina and G. Varga, *Giorn. Batteriol. immunol.* **19**, 850 (1937).
[464] E. Cottingham and C. A. Mills, *J. Immunol.* **47**, 493 (1943).
[465] G. H. Bourne, *Lancet* **246**, 688 (1944).

activity and fragility of these cells. The ascorbic acid content of the exudate varied between 0 and 1.25 mg. per 100 ml. of exudate. They found that the percentage of cells showing phagocytosis has a direct relation to the ascorbic acid content. With levels from 0 to 0.25 mg. per 100 ml. only 30 to 35 % of the cells showed phagocytosis; with 1 to 1.25 mg. per 100 ml. of exudate 80 to 90 % of the cells were active. They also found that the fragility of the cells bore an inverse relationship to the ascorbic acid level of the exudate. Their results are shown graphically in Fig. 3.

The results of the investigations herein cited leave little doubt of a stimulatory effect of ascorbic acid on phagocytic activity.

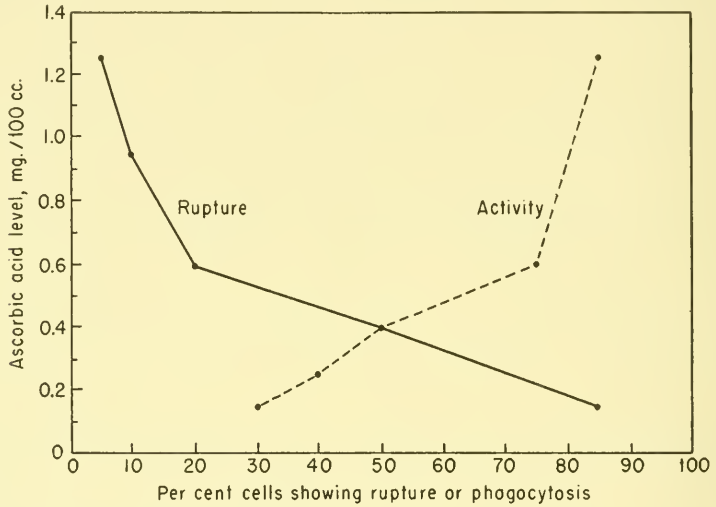

FIG. 3. The relationship between ascorbic acid and phagocytic activity. (W. J. Nungester and A. M. Ames[21].)

b. Virus Diseases

The inhibition of virus growth by ascorbic acid has been reported by several investigators. Jungeblut[466] reported a virucidal action of the vitamin on poliomyelitis virus in monkeys. Synthetic ascorbic acid was less effective than the naturally occurring vitamin, possibly because of associated flavonoids. Lojkin[467] observed that ascorbic acid in concentrations of 0.03 mg. per milliliter caused inactivation of tobacco mosaic virus when the vitamin in the virus solution was oxidized by atmospheric oxygen. The presence of copper stimulated the reaction. Holden and Molloy[468] reported that herpes

[466] C. W. Jungeblut, *J. Exptl. Med.* **66**, 459 (1937).
[467] M. Lojkin, *Contribs. Boyce Thompson Inst.* **8**, 335 (1936).
[468] M. Holden and E. Molloy, *J. Immunol.* **33**, 251 (1937).

virus, incubated with vitamin C at pH 6.0, was found to be inactive when tested intracerebrally in rabbits. Kligler and Bernhoff[469] in experiments with vaccinia virus found that relatively small amounts of ascorbic acid inactivated infective doses of the virus. Glutathione was also effective but somewhat less so. McCarty[470] investigated the mechanism of the virucidal action of ascorbic acid on pneumococcal types, and Klein[471] its action on influenza virus A. They found that the effects of the vitamin are probably produced chiefly by the formation of hydrogen peroxide. Klein has expressed the opinion that "the virucidal activity of ascorbic acid can have no therapeutic application because the presence of catalase in body tissues will destroy the hydrogen peroxide formed during the copper-catalyzed oxidation of ascorbic acid." Other workers in this field tend to accept this view with some reservation, since there may be other substances present in cells which may also react with hydrogen peroxide.[86a, b]

c. Allergic and Anaphylactic Reactions

Ascorbic acid also appears to play a role in these conditions. Hochwald[472] reported finding that the allergic condition is associated with an alteration in blood proteins involving increases in fibrinogen and globulin. Increase in fibrinogen and decrease in the albumin/globulin ratio have been previously mentioned as characteristic of scurvy. Hochwald found that the albumin/globulin ratio was elevated by administration of vitamin C. If 100 mg. of the vitamin was injected into sensitized guinea pigs a few minutes before administering the shocking dose, no anaphylaxis developed. He also reported finding that ascorbic acid had no effect on histamine shock. It was interesting in view of the fact that no characteristic blood protein changes occur in the latter condition. Several other investigators have reported finding that vitamin C makes guinea pigs more resistant to serum shock, especially if administered in advance of the shocking dose.[473-479] Yosikawa[480] stated that vitamin C inhibits allergy only with continued use of rather large doses; smaller doses, in fact, often intensify the allergy. Diehl[481] re-

[469] I. J. Kligler and H. Bernhoff, *Nature* **139**, 965 (1937).
[470] M. McCarty, *J. Exptl. Med.* **81**, 501 (1945).
[471] M. Klein, *Science* **101**, 587 (1945).
[472] A. Hochwald, *Z. ges. exptl. Med.* **97**, 433 (1935).
[473] P. Giroud and A. Giroud, *Compt. rend. soc. biol.* **121**, 1588 (1936).
[474] B. Solomonica, *J. Immunol.* **31**, 209 (1936).
[475] H. Lemke, *Monatschr. Kinderheilk.* **67**, 244 (1936).
[476] G. Ungar, J. L. Parrot, and A. Levillain, *Compt. rend. soc. biol.* **125**, 1015 (1937).
[477] M. Yamamoto, *Oriental J. Diseases Infants* **23**, 11 (1938).
[478] M. Hasimoto, S. Kitamura, and S. Suzuki, *J. Oriental Med.* **28**, 155 (1938).
[479] S. Yokayama, *Kitasato Arch. Exptl. Med.* **17**, 17 (1940).
[480] K. Yosikawa, *Acta Med. Nagasak.* **I**, Ref. 10 (1939).
[481] F. Diehl, *Klin. Wochschr.* **18**, 956 (1939).

ported that anaphylactic shock in guinea pigs with partial vitamin C deficiency is much more severe than it is in normal animals or than in animals on the verge of scurvy. Bronfenbrenner *et al.*[482] studied experimental allergy and its prevention in guinea pigs. They reported finding that ascorbic acid has no influence on the development of sensitivity or prevention of shock, once the animal is sensitive. They suggested that an animal on a diet lacking vitamin C may develop alterations in the intestinal tract and, if already sensitized parenterally, will as a consequence of the vitamin deficiency develop an increased rate of absorption from the intestine, allowing it to absorb the antigen more readily, resulting in shock resembling anaphylaxis produced parenterally. No histological evidence of such intestinal alterations was presented, however.

The fact that several other investigators have been unable to confirm the finding that ascorbic acid reduces anaphylactic reactions in guinea pigs indicates that the problem has not yet been satisfactorily solved.[483-487]

9. Reproduction

Goettsch[488] reported that female guinea pigs deprived of vitamin C maintained a regular estrus rhythm until they began to lose weight. If they received an amount of the vitamin much less than sufficient to protect them from chronic scurvy the cycles continued. Kramer *et al.*[185] observed, however, that when females were kept on a vitamin C-free diet there were no signs of estrus. Harman[127] reported that males of comparable ages succumb to the effects of ascorbic acid deficiency sooner than do females. They became sexually inactive within 3 or 4 days after being put on an ascorbic acid-free diet. The testes were found to leave the scrotum and to return to the body. Normal spermatozoa were not produced, and the entire walls of the seminiferous tubules appeared to be degenerating.

Ingier[4] found that the susceptibility of the female to scurvy is greatly increased during pregnancy although marked symptoms usually do not develop until after parturition. Definite scurvy was produced in the fetus as early as the tenth to fifteenth day after the diet of the mother had been changed. The litters were frequently premature or stillborn and showed retarded growth if vitamin C was lacking. Reyher *et al.*[489] confirmed Ingier's

[482] J. Bronfenbrenner, D. M. Hetler, F. M. Love, and J. M. Burnett, *J. Allergy* **11**, 466 (1940).
[483] W. Schäfer, *Z. Immunitätsforsch.* **91**, 394 (1937).
[484] J. Van Niekirk, *Acta Brevia Neerl. Physiol. Pharmacol. Microbiol.* **7**, 144 (1937).
[485] S. Raffel and R. R. Madison, *J. Infectious Diseases* **63**, 71 (1938).
[486] G. Walther, *Z. ges. exptl. Med.* **105**, 584 (1939).
[487] P. Vallery-Radot, G. Maurie, and M. A. Holtzer, *Compt. rend. soc. biol.* **136**, 281 (1942).
[488] M. Goettsch, *Am. J. Physiol.* **95**, 64 (1930).
[489] P. Reyher, E. Walkhoff, and O. Walkhoff, *Münch. Med. Wochschr.* **75**, 2087 (1928).

results. Kramer *et al.*[185] and Harman[127] made similar observations. It was shown by Farmer *et al.*[364] that primapara guinea pigs were better able to withstand nutritional deficiencies than were the multipara animals.

Harman and Warren[38] observed that the embryos of ascorbic acid-deficient guinea pigs were smaller than the embryos of the same age from normal animals. The internal organs were smaller in the embryos of deficient animals than in normal embryos of the same age. Muscular tissue was differentiated in the 34-day stage of the fetuses of the control animals but was not observed until the 40-day stage in the deficient animals. Interesting cytological differences between the two groups, particularly with reference to cell walls, are described elsewhere in connection with the discussion of individual organs.

Day[490] showed the great importance to both mothers and young (guinea pigs) of an adequate supply of vitamin C in the maternal diet during lactation. In the absence of the vitamin there was marked loss in weight of the mothers, growth of the young was much retarded, and pronounced abnormalities developed in the teeth, jaws, and facial bones in both mothers and young. Supplements of orange juice, cabbage, or lettuce prevented these conditions approximately in proportion to the amount of vitamin C supplied. Unfortunately, no tests were conducted with crystalline ascorbic acid (possibly because of unavailability).

10. Relation to Hormones and Stress

No attempt will be made to cover the extensive developments in this field. Yearly reviews on the subject appear in *Vitamins and Hormones*.[491]

Murray[260] found that the injection of insulin or adrenal cortical hormones did not alter appreciably the disturbed carbohydrate metabolism in scurvy; the glycogen levels remained low, intestinal absorption was low, and blood sugar levels were high after feeding glucose. Adrenaline administered to both normal and scorbutic animals produced higher blood sugar levels in the normal than in the deficient animals. Another observation of particular interest was made by Patterson.[492] He found a hyperglycemic effect of dehydroascorbic acid in rats. Three daily injections of 80 mg. each resulted in an apparent permanent diabetes in animals weighing about 120 g. The mechanism of action, which appears to be similar to that of alloxan, is probably an interference with essential sulfhydryl enzymes in the beta cells of the pancreas. It seems possible that the destructive action may be effected by the rapid production of H_2O_2. Patterson suggested that studies could profitably be made of substances which cause excessive oxidation of ascorbic acid to dehydroascorbic acid in the beta cells because of the pos-

[490] C. D. M. Day, *Indian J. Med. Research* **35**, 185 (1947).
[491] Vitamins and Hormones, Academic Press, New York.
[492] J. W. Patterson, *J. Biol. Chem.* **183**, 81 (1950).

sibility that these compounds may act as precipitating factors in human diabetes.

Giroud and coworkers[493] were the first to report a correlation between the content of the cortical hormone in the adrenal and the ascorbic acid intake. Great interest in the problem was generated by the report of Sayers *et al.*[282] of marked changes in the adrenal ascorbic acid and cholesterol following administration of adrenotropic hormone. Stresses of various kinds have been shown to cause increased adrenal cortical activity correlated with a rapid reduction in the ascorbic acid content of the gland.[178, 494-500] Stepto *et al.*[178] obtained results in studies with guinea pigs which indicated that hypertrophy of the adrenals does not occur until the ascorbic acid-cholesterol ratio falls below 1:500. The degree of stress resulting from the restricted food intake of guinea pigs on a scorbutigenic diet has been shown to be much less than that imposed by the vitamin deficiency in its advanced stage.[182a]

The rapid disappearance of ascorbic acid from the adrenal during periods of activity due to stress has been interpreted by some workers as indicating that ascorbic acid is involved in the synthesis of the cortical hormone,[196, 493, 501] whereas others[502-506] have presented evidence suggesting that it has little or no effect. Pirani[507] has pointed out that, although present evidence indicates that high concentrations of ascorbic acid are unnecessary for adrenal cortical function, there is no indication that the vitamin is not needed at least in small amounts. Even in advanced scurvy small amounts of the vitamin are present, part of it in bound form.[250b] In scurvy, an increased excretion of formaldehydegenic substances has been reported by

[493] A. Giroud, N. Santa, and M. Martinet, *Compt. rend. soc. biol.* **134,** 23 (1940).
[494] H. Selye, *Endocrinology* **21,** 169 (1937).
[495] J. D. Ingle, *Am. J. Physiol.* **124,** 627 (1938).
[496] *Nutrition Revs.* **6,** 143 (1948); **8,** 52 (1950).
[497] M. Thérein and L. P. Dugal, *Rev. can. biol.* **8,** 248, 440 (1949).
[498] M. Thérein, J. Leblanc, O. Heroux, and L. P. Dugal, *Can. J. Research.* **27E,** 349 (1949).
[499] A. Dury, *Am. J. Physiol.* **163,** 99 (1950).
[500] H. Bacchus and C. A. Toompas, *Science* **113,** 269 (1951).
[501] E. H. Herrick, E. R. Mead, B. Egerton, and J. S. Hughes, *Endocrinology* **50,** 259 (1952).
[502] J. W. Jailer and N. F. Boaz, *Endocrinology* **46,** 314 (1950).
[503] H. S. Treager, G. J. Gabuzda, N. Zamchek, and C. S. Davidson, *Proc. Soc. Exptl. Biol. Med.* **75,** 517 (1950).
[504] A. B. Eisenstein and R. E. Shank, *Proc. Soc. Exptl. Biol. Med.* **78,** 619 (1951).
[505] M. Vogt, *J. Physiol.* (*London*) **107,** 239 (1948).
[506] G. A. Hyman, C. Ragan, and J. C. Turner, *Proc. Soc. Exptl. Biol. Med.* **75,** 470 (1950).
[507] C. L. Pirani, *Metabolism* **1,** 197 (1952).

Clayton and Prunty[508] and Nadel and Schneider.[509] These results are of considerable interest, but further study is required to determine more definitely the nature and place of origin of these compounds.

There is some discrepancy in the reported effects of cortisone on the hemorrhagic manifestations of scurvy in guinea pigs. Schaffenburg *et al.*[510] found that cortisone inhibits many of the symptoms of scurvy in the guinea pig though not the capillary hemorrhages, whereas desoxycorticosterone aggravates the condition. Hyman *et al.*[506] found that both ACTH and cortisone prolong life in scorbutic guinea pigs and reduce the tendency to hemorrhage. The two hormones produced similar effects on the scorbutic animal with respect to maintenance of glycogen storage in liver, adrenals, and muscle. As a result of the moderating effect of ACTH on scurvy these workers thought it probable that vitamin C is not necessary for the production of the corticosteroids similar to cortisone. The increased adrenal activity induced by ACTH in the apparent absence of ascorbic acid in the adrenals was interpreted as additional support for this hypothesis. Eisenstein and Shank[504] also found that administration of ACTH delayed the onset of severe scurvy in guinea pigs and increased the mean survival period. They interpreted their results as indicating that vitamin C is not directly involved in the elaboration of adrenal cortical hormones with an oxygen atom at the C^{11} position. Herrick *et al.*[501] in similar studies found that animals not receiving cortisone developed painful joints and degenerating bones and testes and had a survival time of 12 to 19 days, whereas those receiving the hormone developed no painful joints, their bones and testes had nearly normal structure, and they had a survival time of 17 to 45 days. In a continuation of these studies Hughes *et al.*[196] investigated the effects of ACTH, cortisone, and desoxycorticosterone on the development of "arthritic" lesions in scorbutic guinea pigs and found that desoxycorticosterone and ACTH appeared to aggravate the arthritic condition, and that cortisone suppressed it, a result which suggested to these workers the possibility that "vitamin C may be essential in the production of the oxy type of adrenal-cortical hormones." Although much study has been made of the relation of vitamin C to the adrenals, the time has not arrived for drawing definite conclusions as to its role in the production of corticosteroids. However, the burden of proof appears to rest on those who hold that it has a positive effect.

[508] B. E. Clayton and F. T. C. Prunty, *Brit. Med. J.* **2**, 927 (1951).

[509] E. M. Nadel and J. J. Schneider, *Endocrinology* **51**, 5 (1952).

[510] C. Schaffenburg, G. M. C. Masson, and A. C. Corcoran, *Proc. Soc. Exptl. Biol. Med.* **74**, 358 (1950).

IX. Effects of Deficiency in Human Beings

RICHARD W. VILTER

A. INTRODUCTION

The winter had been unusually cold and unpleasant. Joe S., a bachelor and pensioner at the age of 65, had stayed almost entirely in his room except for occasional visits to the store down the street for hamburger, frankfurters, eggs, condensed milk, bread, and coffee. He had beer almost daily in the bar downstairs, but it had been at least six months since he had had any fruits or vegetables. Oranges and tomatoes were fit only for pigs in his estimation, and since he usually ate alone, he prepared only those foods that were simplest and cheapest. In March, he noticed that he was growing weaker and somewhat short of breath. In April, though the weather was mild, he seldom went out because of fatigue and apathy. He was surprised one evening to see tiny red spots around his ankles and an unexpected bruise or two around his knees. There was deep, dull, aching discomfort in his legs. After several days more he had become too weak even for his simple routine and remained in bed most of the time. Much larger bruises had appeared on his legs. The right calf was completely discolored and had become swollen, hard, and tender. An old ulcer on his right ankle had broken down and was infected. By the first of May, the bruises had extended to his thighs and abdomen and his gums were sore and swollen. The tissues bled when he tried to chew what little food the neighbors brought in. His color became sallow, his eyes tinged with yellow, and his weakness so great that he could no longer get out of bed. At the insistence of neighbors, the city hospital amulance was called and Joe S. was carried to the admitting department. He might have been sent to the vascular service or the dermatology ward had not a medical resident spied him and made the correct diagnosis. Joe S. had scurvy.

He had a disease that was described in the Ebers Papyrus discovered at Thebes about 1500 B.C.; a disease well known to Hippocrates (460–370 B.C.), who reported that large numbers of men in the army suffered from pains in the legs and gangrene of the gums accompanied by loss of their teeth; a disease which decimated the armies of the Crusaders more than the Saracans did and which spread over Northern Europe and the low countries with the black plague during the Renaissance. It was not described on shipboard until 1492 when seafarers set out on long voyages. Magellan lost nine-tenths of his crew as a result of scurvy; Vasco da Gama, 100 out of 160 men; and Lord Anson four-fifths of a crew of 1200 men.[1, 2] Jacques

[1] R. H. Major, Classic Descriptions of Disease with Biographical Sketches of the Authors. Charles C Thomas, Springfield, Ill., 1945.

[2] A. F. Hess, Scurvy, Past and Present. Lea and Febiger, Philadelphia, 1920.

Cartier's men had scurvy in Newfoundland in 1536 to the extent of 100 casualites out of 110 men. A historian in his group wrote: "An unknown sickness began to spread itself among us after the strangest sort that ever was heard or seen. Some did lose all their strength and could not stand on their feet. Then did their legs swell, their sinnows shrinke as black as any cole. Others also had all their skins spotted with spots of blood of a purple color. Then did it ascent up to their ankles, thighes, shoulders, arms and necks; their mouths became stinking; their gums so rotten that all the flesh did fall off even to the roots of the teeth which did almost all fall out. With such infection did this sickness spread itself in our three ships that about the middle of February of 110 persons that we were, there were not 10 whole."

"That day Phillip Rougemont died being 22 years old and because the sickness was to us unknown, our captain caused him to be ripped to see if by any means possible we might know what it was, and so seek means to save and preserve the rest of the company. He was found to have his heart white, but rotten, and more than a quart of red water about it. His liver was indifferent fair, but his lungs black and mortified. That done as well as we could, he was buried." The Indians showed them how to make a decoction of swamp spruce, and from this mixture the rest were saved.[3]

In the sixteenth century, scurvy was described by Claus Magnus, Echtius, and Wierus. Ponsseus referred to the therapeutic use of scurvy grass, watercress, and oranges.[4] By 1600, lime juice was used experimentally in the British Navy, especially on ships of the East India Company. In 1734, Bachstrom gave the first clear account of the relationship of a deficiency in fresh fruits and vegetables to scurvy.[2] In 1753, James Lind, a ship surgeon, published his epochal treatise on scurvy and his controlled experiments demonstrated the spectacular curative effect of oranges and lemons.[4] By 1795, lemon juice was a regular ration in the British Navy, ounces 1 daily after 10 days at sea. In 1854, a similar regulation was put into effect in the British Merchant Marine. In spite of the toll taken by scurvy in the war between the States, there was no provision in the United States Army for antiscorbutic rations until 1895. Scurvy continued to take its toll through World War I.

Infantile scurvy was described by Glisson in 1650,[5] but he confused the clinical picture with rickets. This misinterpretation continued until Sir Thomas Barlow, in 1883, differentiated infantile scurvy from rickets and

[3] H. P. Biggar, The Voyages of Jacques Cartier. Publications of Public Archives of Canada, No. 11, p. 204, 1924.

[4] J. Lind, Treatise on Scurvy, 2nd ed., A. Millar, London, 1757.

[5] F. Glisson, De Rachitide sive Morbo Puerti qui vulgo, "The Rickets," dicitur. London, 1650. Quoted by F. F. Tisdall and N. Jolliffe, Vitamin C. Malnutrition and Scurvy in Clinical Nutrition, pp. 586–601. Paul B. Hoeber, New York, 1950.

demonstrated its similarity to scurvy in adults.[6] Since that time a synonym for scurvy has been "Barlow's disease." Pasteurization of milk greatly increased the incidence of infantile scurvy until supplementation of infant diets with orange juice became popular.

Hopkins suggested that scurvy was a deficiency disease in 1906.[7] In 1907, Holst and Frölich produced scurvy in guinea pigs by feeding them restricted diets.[8] Not until 1928, however, was "hexuronic acid" isolated by Szent-Györgyi[9] and shown to be identical with vitamin C by Waugh and King, who isolated it from lemons.[10] For a time vitamin C was called cevitamic acid, but in 1939 the Council on Pharmacy and Chemistry of the American Medical Association adopted the name ascorbic acid as the official designation.

During the past ten years biochemists and clinicians have been probing into the chemical relationship of ascorbic acid to body chemistry and function. Much has been learned. Even more remains to be learned. In spite of this accumulation of knowledge in the prevention and treatment of scurvy dating back to the seventeenth century, this malady continues to take its toll and even now sporadic cases of scurvy in infants and adults still appear in the admitting departments of most large municipal hospitals, just as Joe S. appeared. Poverty, ignorance, and prejudice account for most of them.

B. CLINICAL ASPECTS

I. Incidence

Though there is undoubtedly a great deal of subclinical ascorbic acid deficiency, clinical scurvy is a rare disease in the United States of America and in most of the civilized world today. It occurs for the most part in urban areas and slum sections, and examples of the disease appear from time to time in the wards of most municipal hospitals (see Fig. 4). One peak of incidence occurs between the ages of 6 and 12 months[11] in infants whose processed milk formulas are unsupplemented with citrus fruits or vegetables, or because they refuse or spit out the orange juice offered them. The disease seldom if ever occurs before the age of 4 months and only occasionally after 18 months. Breast-fed infants are protected if their mothers are well nourished. Another peak occurs in the middle and old age groups.

[6] T. Barlow, On Cases Described as "Acute Rickets" which are Probably A Combination of Scurvy and Rickets. *Medico-Chirurg. Trans.* (*London*) **LXVI**, 1959 (1883); reprinted in *Arch. Disease Childhood* **10**, 223 (1935).

[7] F. G. Hopkins, *Analyst* **31**, 385 (1906).

[8] A. Holst and T. Frölich, *J. Hyg.* **7**, 634 (1907).

[9] J. L. Svirbely and A. Szent-Györgyi, *Biochem. J.* **26**, 865 (1932).

[10] W. A. Waugh and C. G. King, *J. Biol. Chem.* **97**, 325 (1932).

[11] I. Dogramaci, *New Engl. J. Med.* **235**, 185 (1946).

Bachelors and widowers who live alone, cook for themselves, or eat alone in restaurants are particularly prone to develop the disease. In this age group, the disease occurs predominantly in males. The elderly woman almost always has someone looking after her, and fruits and vegetables occupy a more prominent place in her diet.

Most cases of scurvy in adult persons occur in the spring, with a second but smaller peak in the early fall months along with other deficiency diseases. In 241 infants, the peak occurred in August and September.[11] Spring and fall fluctuations in incidence hold true, whether the disease occurs because of a primary dietary inadequacy in ascorbic acid or is conditioned

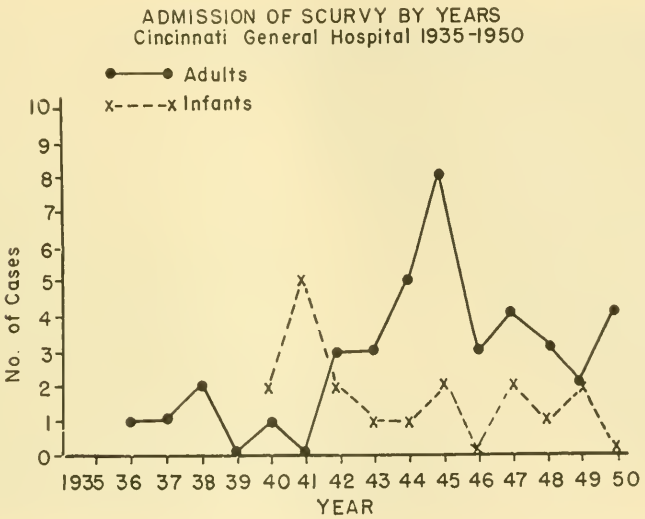

Fig. 4

by the increased requirements for the vitamin imposed by hypermetabolism, chronic infection, or the increased loss which occurs in persons with chronic diarrhea (see Fig. 5).

2. Prodromata

It is difficult to say at what stage of ascorbic acid tissue depletion the first deleterious effect occurs. Chemical processes within the cells are probably impaired long before symptoms or physical manifestations occur. It has been alleged that chronic gingivitis, pyorrhea, and bleeding gums are the result of chronic mild ascorbic acid depletion[12] based on low plasma ascorbic acid levels and tissues half saturated with the vitamin in many of

[12] W. J. Linghorne, W. G. McIntosh, J. W. Tice, F. F. Tisdall, J. F. McGreary, T. G. H. Drake, A. V. Greaves, and W. M. Johnstone, *Can. Med. Assoc. J.* **54**, 106 (1946).

the affected persons. However, therapeutic trials with ascorbic acid have been disappointing, and, furthermore, low plasma levels are not a satisfactory index of tissue depletion, since tissue levels of 0 mg. % are usually reached before clinical scurvy occurs. In experimental ascorbic acid depletion, gum lesions did not occur,[13] occurred rarely,[14] or in more prolonged studies occurred only after the tissue stores of ascorbic acid were exhausted and other scorbutic lesions had been present for many weeks.[15] In many clinical examples of the disease studied by the author, gum lesions occurred

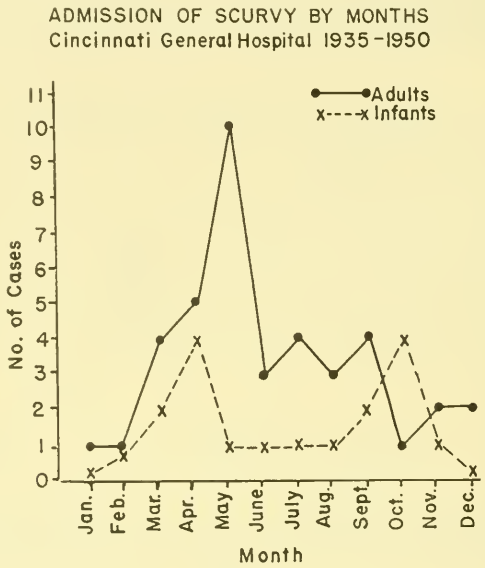

FIG. 5

only after many other symptoms and signs had appeared. It is doubtful whether chronic gingivitis is often related to vitamin C deficiency.

The first symptoms reported in experimentally induced scurvy[13, 14] and by patients with the disease are weakness, easy fatigue, and listlessness. These are followed quickly by shortness of breath and aching in bones, joints, and muscles of the extremities. This aching is worse at night. Appetite is moderately reduced, but most patients continue to eat well until swollen painful gums prevent mastication. The skin usually becomes dry and rough, dingy, and brown from increasing pigmentation. In a human subject on a diet free of ascorbic acid but adequate in other nutrilites,

[13] J. H. Crandon, C. C. Lund, and D. B. Dill, *New Engl. J. Med.* **223**, 353 (1940).
[14] M. Pijoan and E. L. Lozner, *Bull. Johns Hopkins Hosp.* **75**, 303 (1944).
[15] H. A. Krebs, R. A. Peters, K. H. Coward, L. W. Mapson, L. G. Parsons, B. S. Platt, J. C. Spence, and J. R. P. O'Brien, *Lancet* **I**, 853 (1948).

fatigue and poor performance on a treadmill appeared after 90 days and when the white blood cell ascorbic acid level had reached 4 mg. %.[13]

3. Clinical Deficiency State in the Adult

The first clinical manifestation is the perifollicular hyperkeratotic papule (see Fig. 6), which occurs on the legs and buttocks and later on the arms and back. The hairs become fragmented, coiled, and buried in the lesions. These closely resemble the follicular hyperkeratoses of vitamin A deficiency and appear after about 4 months of complete ascorbic acid deprivation. Erythema and then purpura appear around these hair follicles, producing

Fig. 6. Perifollicular hemorrhages on the leg of a boy, age 16, with scurvy.

the distinctive early sign of scurvy, the perifollicular hemorrhage, which has appeared in experimental subjects after 5½ months of ascorbic acid deprivation and after the white cell-platelet ascorbic acid level had been zero for 1½ months.[13, 14, 15] This is not a specific manifestation, however. Morphologically identical lesions have been seen in patients with bacterial endocarditis and follicular hyperkeratosis.

Purpura appears on the lower extremities first. It spreads upward and has a predilection for areas around joints and parts frequently traumatized. Minute purpuric spots may be seen along scratch marks, where garters or brassieres constrict or where shoes exert pressure. The ecchymoses become larger, coalesce, and may involve the skin of the entire extremity (see Fig. 7). Hemorrhages deep in muscle occur, particularly in calf, thigh, and forearm, causing painful brawny induration of the surrounding tissues.

If the overlying skin areas are not already involved in the hemorrhagic state, they become discolored (yellowish-green) from the underlying hemolyzed blood. Phlebothromboses are common in these areas, thereby increasing induration, swelling, and pain.

In 1752, James Lind[4] described scorbutic skin lesions in the following manner, which can scarcely be improved now. "The skin is dry, feverish, rough and covered with several reddish, bluish or rather black and livid spots equal with the surface of the skin resembling an extravasation under it as it were from a bruise. These spots are of different sizes, from the bigness of a lentil to that of a hand's breadth and larger. . . . They are usually to

Fig. 7. Extensive confluent ecchymoses on the legs of a patient with scurvy. The ecchymotic areas were brawny and indurated.

be seen chiefly on the legs and thighs, often on the arms, breast and trunk of the body; but more rarely on the head and face."

Hemorrhages may occur into joints, causing swelling, pain, local heat, and tenderness. Large joints subject to frequent trauma are usually affected. The skin overlying the joint becomes discolored a dirty greenish-yellow from the hemolyzed blood in and around it. Other signs of scurvy may be so few that such patients are admitted to the hospital with the diagnosis of rheumatoid arthritis. The incorrectness of this diagnosis becomes apparent when the joint is aspirated and fresh or hemolyzed blood is obtained. Hemorrhages of the splinter type appear under the nail beds lying side by side parallel to the long axis of the finger (see Fig. 8). They form a crescent near the distal end of the nail. When they occur in scurvy, there are usually many more of them than are ever seen in bacterial endocarditis.

FIG. 8. Splinter hemorrhages arranged in a semicircular lattice involving the nail beds in a patient with scurvy.

FIG. 9. Swollen blue-red gingival lesions in a patient with severe scurvy.

As the deficiency of ascorbic acid becomes more severe, the gums become swollen, blue-red, spongy, and very friable (see Fig. 9). They bleed with slight trauma, though the hemorrhages are usually small and stop quickly. At first the gum lesions are small purpuric spots at the gingival margin of the interdental papillae. Bleeding into the tissues and thrombosis of small

FIG. 10. Infarcted gangrenous interdental papillae and gingival margins in a patient with severe scurvy.

vessels occur, producing the swelling and the blue-red color. The swelling may become so great that gum tissue completely encases and hides the teeth. The swollen devitalized gum becomes secondarily infected, and the breath becomes foul and salivation excessive. Ultimately these lesions progress to complete infarction and gangrene of the gums from the tooth roots to the gingival margin (see Fig. 10). The gum lesions occur only when natural teeth, worn-down snags, or embedded and hidden tooth roots remain in the mouth (see Fig. 11). A scorbutic patient who is apparently

edentulous but who has a focal heaped-up blue-red gum lesion will be found to have a hidden tooth root under the lesion if x-rays of the jaws are made. James Lind described the gum lesions also in vivid terms. "The gums become itchy and swell and are apt to bleed upon the gentlest friction. The breath is then offensive. . . . The gums appear of an unusual livid redness, are soft and spongy, and become afterward extremely putrid and fungous." Though hemorrhages occur in the mucous membranes elsewhere in the mouth, the typical gum lesion does not spread from the gingiva. Such blue-

FIG. 11. Scorbutic gingival lesion around a solitary tooth snag. The edentulous gum was normal.

red, swollen, friable hemorrhagic gums occur in few other diseases, though toxic reactions to dilantin and acute monocytic leukemia may include gum lesions which mimic scurvy. The red, ulcerated atrophied gums of chronic non-specific gingivitis are sufficiently different in appearance so that differentiation from scurvy is easy.

The teeth loosen in the alveolar bone. They can be moved easily with the finger, and many scorbutic patients have performed their own extractions or have had teeth fall out while they were attempting to eat. Once the tooth is out the lesion recedes slowly, but complete healing is delayed until ascorbic acid is supplied. Lesions of the gums have occurred late in the course of human scurvy induced experimentally,[14, 15] and slight fragmenta-

tion of the lamina dura of the teeth in dental x-rays has been observed once in such a person.

Old ulcers and scars become red and break down. New wounds fail to heal, or when apparently healed break open with stretching. These observations, which have been recorded since the Middle Ages, are amply confirmed by tests of wound healing, tensile strength of scars, and histologic appearance of biopsy material from the region of the scar in persons with induced scurvy.[13, 15-17] In scorbutic subjects such experimental wounds open after 8 to 10 days, revealing an unorganized blood clot in the base. This difficulty with healing is most evident within the first 10 days after the wound is made. During this time the tensile strength of the fascia is reduced 50% and of the skin 30% below control levels.[16] These abnormalities of the healing process occur only after clinical scurvy has appeared or when tissue levels, white blood cell levels, or saturation tests indicate very severe depletion of ascorbic acid from the tissues. A low plasma level is not sufficient indication that a wound may fail to heal because of ascorbic acid deficiency.

In support of the relationship of ascorbic acid to tensile strength of wounds, this vitamin appears in very high concentration around abdominal wounds during the healing process in subjects well saturated with it. In depleted subjects, little if any increase occurs in the healing tissues, and when the tissue level remains zero, the tensile strength of the wound is impaired.[17]

Petechial hemorrhages occur in the viscera, but massive hemorrhages are rare. A small bloody pericardial effusion may be found. It is unusual to find more than a few erythrocytes in the urine or more than a positive guaiac test for occult blood in the stool. External hemorrhage is seldom sufficient in the adult to cause anemia.

The sclerae become icteric, the skin a grayish-yellow cadaveric color. Fever of 101° to 102° is common is severe cases. The nail beds and lips become cyanotic due to stasis of blood. The blood pressure gradually falls, Cheyne-Stokes type of respiration appears, the patient becomes more dyspneic and suffers from precordial oppression. If treatment is not forthcoming, convulsions, shock, and death may occur suddenly.

4. Scurvy in Infancy and Childhood

Infantile scurvy differs in certain respects from the disease in adult persons principally because the growing bones of infants react differently

[16] J. A. Wolfer, C. J. Farmer, W. W. Carroll, and D. O. Manshardt, *Surg., Gynecol. Obstet.* **84**, 1 (1947).

[17] M. K. Bartlett, C. M. Jones, and A. E. Ryan, *New Engl. J. Med.* **226**, 469, 474 (1942).

to ascorbic acid deficiency than do the mature bones of adults. The prescorbutic infant becomes anorexic and listless for only a few days before lesions appear. With the onset of scurvy, the infant lies with legs drawn up on its abdomen. It cries when it is touched, especially when its legs or arms are moved or when it is lifted by its feet to change its diaper. Extremely tender swellings may be felt at the ends of the long bones due to

FIG. 12. Healed scorbutic rosary in a boy who had had scurvy off and on since infancy. The sharp edges at the costochondral junctions are easily visible.

hemorrhage under the periosteum of the shaft, but not involving the joints, and edema in the tissues overlying the hemorrhages. The lower end of the femur and the upper end of the humerus are most often affected. In very severe cases epiphysial separations may occur, which may be felt most commonly at the costochondral junctions. The sternum may sink slightly inward, leaving a sharp elevation on the rib side of the junction. This has been called the scorbutic rosary (see Fig. 12). Purpura occurs in the skin in only one-third of the cases, and, as in the adult person, these hemorrhages are conditioned by slight trauma. If teeth have erupted, hyperplastic blue-red swollen gum lesions appear most often around the upper

incisors. Red blood cells may appear in urine, stool, and spinal fluid. Retrobulbar, subarachnoid, and intracerebral hemorrhages may occur. Dyspnea, cyanosis, convulsions, and death follow in rapid progression if treatment with ascorbic acid is delayed.

C. LABORATORY DIAGNOSTIC AIDS

1. Roentgenological Study of the Bones

The earliest manifestations of scurvy in the infant appear in the bones and can be demonstrated by x-ray techniques.[18] These lesions do not occur in the adult patient, and therefore the x-ray is of little help in the diagnosis of adult scurvy. In the diagnosis of infantile scurvy it is of tremendous importance.

The earliest roentgenologic manifestations of infantile scurvy are usually noted at the ankles first and then at the wrists, but at either area they are radiologically identical. A defect appears at the anterior corner of the tibia or at the outer corner of the lower end of the radius. At first the cortex in these areas merely has a fuzzy appearance and there is slight rarefaction of neighboring cancellous bone, making the corners indistinct. Later a cleft or crevice or the extrusion of bony spicules appears at the outer edge of the cortex just underneath the lattice or epiphyseal line. This is called "the corner sign." The fibula and the ulna are less regularly affected, but when the lesion appears in these sites it is of the same general type. If anterior and posterior clefts are very near to each other, epiphyseal separation may occur and the epiphyseal side of the lattice appears to be curved and rests against the end of the shaft.

The most distinctive x-ray sign of scurvy is the dense white line lying across the end of the shaft. It is composed of calcified cartilaginous matrix which is not destroyed in the normal fashion. Much of its dense appearance is due to the rarefaction of bone underneath it, through which the clefts, fractures, and separations occur (see Pathology and Pathologic Physiology).

The cortex of the bones is generally thinned out, giving a "ground-glass" appearance. The thinned-out epiphyses with rims of heavy calcification at the outer margins give the appearance of "halos" (see Fig. 13). The costochondral junctions become broader, and the lattice is irregular. Fractures occur just under the lattice, the ribs remain rigid, and the sternum sinks back, producing the scorbutic rosary.

Hemorrhages which occur under the periosteum and which arise from the fractures through the rarefied zone cannot be visualized by x-ray except as soft tissue shadows at the ends of the bones which do not involve the

[18] E. A. Park, H. G. Guild, D. Jackson, and M. Bond, *Arch. Disease Childhood* **10**, 265 (1935).

joints. After ascorbic acid has been administered, calcification of these rapidly organizing hematomas takes place, and then they cast a distinctive x-ray shadow of lamellated appearance (see Fig. 14).

FIG. 13. Roentgenogram illustrating the scorbutic white line, halo epiphysis, and corner sign in an infant with scurvy.

FIG. 14. Calcification occurring in the region of subperiosteal hemorrhage in an infant with scurvy 2 weeks after treatment with ascorbic acid. The femoral epiphysis is displaced. (Courtesy Dr. Frederick Silverman, Children's Hospital, Cincinnati, Ohio.)

No other changes due to ascorbic acid deficiency in organs or tissues of scorbutic infants or adult persons can be demonstrated by x-ray techniques.

2. Tests for Capillary Fragility

The Rumpel-Leede test[19] almost always demonstrates an abnormality in the state of the capillaries in naturally occurring scorbutics, although this test does not necessarily become positive in induced scurvy in human subjects.[13] It is performed by applying a blood pressure cuff above the elbow, marking out a circle 2.5 cm. in diameter on the skin just below the anticubital fossa, inflating the cuff to a pressure midway between the systolic and diastolic pressure of the patient, and maintaining this pressure for 15 minutes. Three minutes after release of this pressure, the number of petechiae which have appeared in the circle are counted. One to ten are considered normal, ten to twenty are borderline, and over twenty are definitely abnormal. This test has been extended and modified by Göthlin.[20] A similar test which utilizes negative pressure exerted through a cup has been described by Dalldorf.[21] In scurvy these variations give the same abnormal reactions as does the Rumpel-Leede test. These tests are not specific for scurvy. They are abnormal in any disease which increases capillary fragility and permeability.

3. Ascorbic Acid Levels in Blood and Urine

The plasma level of ascorbic acid falls too rapidly after the vitamin is removed from the diet to be of much diagnostic value, though Farmer and Abt[22] felt that levels below 0.5 mg. % were compatible with scurvy. However, in the experiment of Crandon *et al.*,[13] which has been confirmed many times,[14, 15, 23, 24] the level of ascorbic acid in the plasma fell to zero after 41 days of ascorbic acid deprivation, whereas signs of scurvy did not appear for 134 days. Therefore, the plasma level indicates only the state of ascorbic acid nutrition for the preceding few weeks. A level of 0 mg. % is compatible with but not diagnostic of scurvy. If ascorbic acid has not been administered, any amount found in the plasma beyond the limits of accuracy of the method is incompatible with a diagnosis of scurvy.

Ascorbic acid almost disappears from the urine even before the level in the plasma is zero. Therefore a low level of ascorbic acid in the urine has no more significance than a low plasma level. This is true of single or twenty-four-hour urine collections.

The ascorbic acid level in the whole blood has more diagnostic significance. The level reaches zero after 87 days of ascorbic acid deprivation.

[19] C. Leede, *Münch. med. Wochschr.* **58**, 1673 (1911).
[20] G. F. Göthlin, *J. Lab. Clin. Med.* **18**, 484 (1933).
[21] G. Dalldorf, *Am. J. Diseases Children* **26**, 794 (1933).
[22] A. F. Abt and C. J. Farmer, The Vitamins, p. 411. American Medical Association, 1939.
[23] H. Rietschel and J. Mensching, *Klin. Wochschr.* **18**, 273 (1939).
[24] M. Pijoan and E. L. Lozner, *New Engl. J. Med.* **231**, 14 (1944).

The level in the white cell-platelet layer (the buffy coat), normally 25 to 38 mg. per 100 g., reaches zero after 121 days of deprivation at approximately the same time that scorbutic lesions appear. It is of the greatest diagnostic significance.

4. Saturation and Tolerance Tests

Experience at the Cincinnati General Hospital indicates that persons with clinical scurvy will not have any ascorbic acid demonstrable in the plasma until 1800 to 2000 mg. of this substance have been administered by mouth in divided doses of 100 mg. five times a day. The plasma is drawn each morning before the first dose of ascorbic acid. This is essentially a plasma tolerance test. Other similar tolerance tests have been used previously. Kadji *et al.* introduced the concept of the ascorbic acid index.[25] This index is 100 times the product of the fasting plasma ascorbic acid value and the increase in plasma ascorbic acid 4 hours after intramuscular injection of 200 mg. of the vitamin in infants or 500 mg. in children or adults. They report that in active scurvy the index is below 0.8. In persons with very deficient reserves the index is between 0.9 and 6.0. In normally saturated subjects the index is over 10. Unfortunately, most patients with severely depleted ascorbic acid reserves have plasma ascorbic acid levels of 0 mg.%. Methods for ascorbic acid are not accurate between zero and 0.1 mg.%. Therefore, such patients will have indices of zero whether they have scurvy or not, and the index is no more significant than the fasting plasma level.

Saturation tests which depend upon changes in the urine and plasma ascorbic acid levels after an appropriate test dose have been used more commonly. Such saturation tests have employed the following techniques:

1. Measurement of the 24-hour urinary excretion of ascorbic acid after the oral administration of a standard test dose (5 mg. per pound of body weight).[26]

2. The administration of a standard oral dose of ascorbic acid (15 mg. per kilogram of body weight), and the determination of the blood plasma and urinary ascorbic acid each hour for 5 hours with the patient in a fasting state.[16, 27] In severely depleted or scorbutic patients, the plasma level rises to about 0.4 mg.% and falls rapidly back toward zero. The urine shows little if any ascorbic acid.

3. The measurement of the rate of plasma ascorbic acid drop and urinary excretion in 5 hours following the intravenous administration of a test dose of 1 g. of ascorbic acid to adult patients.[28]

[25] L. Kadji, J. Light, and C. Kadji, *J. Pediat.* **15**, 197 (1939).
[26] M. A. Abbasy, L. J. Harris, S. N. Ray, and J. R. Marrach, *Lancet* **II**, 1399 (1935).
[27] J. F. Rinehart and L. D. Greenberg, *Ann. Internal Med.* **17**, 672 (1942).
[28] I. S. Wright, A. Lilienfeld, and E. MacLenathen, *Arch. Internal Med.* **60**, 264 (1937).

4. Administration of 100 mg. of ascorbic acid by intravenous injection and determination of the urinary excretion during a period of 3 hours. A normal, well-saturated person usually will excrete 50% of the test dose; a depleted person will excrete 15% or less of the test dose; and a scorbutic person will excrete less than 5% of the test dose.[29]

These tests indicate only that a person's tissues are saturated or unsaturated. Since the kidney will reabsorb most of the vitamin when the tissues are at all unsaturated, the test cannot give satisfactory evidence of the degree of unsaturation. These tests cannot make or confirm a diagnosis of scurvy. The results can be only compatible or incompatible with such a diagnosis.

5. Intradermal Test with Dichlorophenolindophenol

This test, using the rate of disappearance (i.e., reduction) of 0.01 ml. of a solution of 2,6-dichlorophenolindophenol (2 mg. to 4.9 ml. of water) injected intradermally, has been proposed as an indicator of vitamin C tissue saturation. There are too many reducing agents other than vitamin C in the skin to allow for any accuracy in diagnosis of ascorbic acid deficiency by this method.[30, 31]

6. Tests for "Tyrosyl Derivatives"

Sealock and Silberstein[32] showed that homogentisic acid, *p*-hydroxyphenylpyruvic acid, and *p*-hydroxyphenyllactic acid are excreted by scorbutic guinea pigs when the animals are fed tyrosine. These intermediate metabolites are excreted by ascorbic acid-deficient premature infants[33] and scorbutic adult human beings also[34] when given tyrosine. Infants are fed 1 to 5 g. and adults 15 to 20 g. of tyrosine daily. Twenty-four-hour urine specimens are analyzed for the compounds mentioned above by the method of Medes,[35] and for reducing substances, mainly *p*-hydroxyphenylpyruvic acid, by the reduction of phosphomolybdic acid. After the administration of vitamin C, the amount of "tyrosyl derivatives" excreted is greatly reduced. There is considerable question whether the administration of folic acid will cause a similar diminution in the tyrosyl derivatives in scorbutic human beings.[36, 37] Apparently where such a diminution has been

[29] E. P. Ralli and S. Sherry, *Medicine* **20**, 251 (1941).
[30] H. G. Rapaport and S. Miller, *J. Pediat.* **15**, 503 (1939).
[31] H. G. Poncher and C. H. Stubenrauch, Jr., *J. Am. Med. Assoc.* **111**, 302 (1938).
[32] R. R. Sealock and H. E. Silberstein, *J. Biol. Chem.* **135**, 251 (1940).
[33] S. Z. Levine, E. Marples, and H. H. Gordon, *J. Clin. Invest.* **20**, 199 (1941).
[34] W. F. Rogers and F. H. Gardner, *J. Lab. Clin. Med.* **34**, 1491 (1949); **28**, 806 (1949).
[35] G. Medes, *Biochem. J.* **26**, 917 (1932).
[36] C. D. Govan, Jr., and H. H. Gordon, *Science* **109**, 332 (1949).
[37] J. E. Morris, E. R. Harpur, and A. Goldbloom, *J. Clin. Invest.* **29**, 325 (1950).

accomplished by folic acid the dose has been exceedingly large, averaging 13 mg. per kilogram of body weight per day.

7. Other Laboratory Tests

The electrolytes in the plasma, e.g., sodium, potassium, calcium, phosphorus, and carbon dioxide content, are not disturbed specifically by the scorbutic process. However, debility and severe malnutrition of many scorbutic patients may cause sodium and potassium depletion and metabolic acidosis. Urea nitrogen and blood sugar levels are not specifically affected, and alkaline phosphatase levels are reported to be normal or low.[11]

Twenty-four-hour urinary 17-ketosteroid levels are low in normal or subnormal but in the same range as in most severely malnourished persons; 11-oxysteroid levels have not been measured.

Urobilinogen in urine and stool is increased, and the abnormal amount of bilirubin which appears in the plasma is for the most part of the indirect or slow reacting type. These abnormalities in bile pigment production and excretion are probably due in part to the hemolysis of extravasated blood. However, the amount of jaundice and bilirubinemia do not correlate well with the number and severity of the ecchymoses, and the suggestion has been made that intravascular hemolysis or an abnormality in pigment utilization may occur also.[38]

A few erythrocytes usually are found in the urine and spinal fluid and the stool may give a positive test for occult blood with guaiac reagent. Frank urinary or gastroenteric tract bleeding or hemorrhages into the brain and subarachnoid space are rare.

8. The Hematopoietic System

Most patients with scurvy have anemia. The type and etiology vary. In infancy and childhood the anemia is usually microcytic and hypochromic, but it may be macrocytic or normocytic normochromic.[39, 40] The hypochromic anemia is due principally to a deficiency of iron in the infant's diet and in the mother's diet during the infant's intra-uterine life. Powdered milk formulas deficient in ascorbic acid are also deficient in iron, and the iron deficiency in the infant whose hemoglobin mass is expanding rapidly takes precedence over all other deficiencies. The large hemorrhages into the tissues, particularly the subperosteal hemorrhages and recent external bleeding, contribute to the anemia and tend to make it normocytic normochromic. Infection, if present, accentuates this tendency. As will be seen

[38] R. W. Vilter, R. M. Woolford, and T. D. Spies, *J. Lab. Clin. Med.* **31**, 609 (1946).
[39] W. W. Zuelzer, L. Hutoff, and L. Apt, *Am. J. Diseases Children* **77**, 128 (1949).
[40] L. G. Parsons and W. C. Smallwood, *Arch. Disease Childhood* **10**, 327 (1935).

from studies in adult scurvy and the megaloblastic anemia of infancy, ascorbic acid deficiency probably contributes to the anemia also and, because of its relationship to the metabolism of folic acid, tends to make the anemia slightly macrocytic. The type of anemia which one finds in a scorbutic child depends upon which metabolic factor is most critically needed.

The etiology of the megaloblastic anemia of infancy is related indirectly in many cases to vitamin C deficiency. This type of anemia, first described by Veeneklaas in 1941[41] and by Zuelzer and Ogden[42] in 1946, occurs most commonly in infants fed dried milk formulas unsupplemented by foods rich in vitamin C. It occurs at the same age that infantile scurvy is most prevalent, and at least 25% of infants with megalobalstic anemia have scurvy also. The infant loses its appetite, becomes weak and apathetic, and anemia develops rapidly. It may be macrocytic; more commonly it is normocytic and normochromic though it may be hypochromic. The megalobasts and bizarre metamyelocytes in the bone marrow typical of a deficiency of one of the erythrocyte maturation factors are frequently the only distinctive diagnostic feature. The anemia does not respond to the administration of ascorbic acid alone, and rather poorly if at all to the administration of vitamin B_{12}. It responds dramatically to folic acid medication.

The probable relationship of this type of anemia to ascorbic acid deficiency has been demonstrated by May and his coworkers[43] by a series of experiments on monkeys fed the same milk diets which infants with megaloblastic anemia had been eating. These diets were deficient in folic acid and ascorbic acid. Megaloblastic anemia usually follows the appearance of scorbutic lesions within a week or two. Either folic acid or ascorbic acid supplements will prevent it, although when folic acid supplements alone are given, scurvy and normocytic anemia occur. Folic acid treatment after the anemia has occurred will rapidly convert the megaloblastic marrow to a normoblastic one and induce reticulocytosis and an erythrocyte response. Ascorbic acid administration alone cures the scurvy and induces reticulocytosis and remission in the anemia, although normalization in the marrow does not occur as rapidly as after folic acid therapy.

Vitamin B_{12} alone does not have any therapeutic effect, but in combination with ascorbic acid it is said to have as dramatic an effect as folic acid on the megaloblastosis and anemia. There is no clear-cut evidence, however, that the improvement is not due to the ascorbic acid alone. Folinic acid is reported to be effective in smaller doses than folic acid.[44]

[41] G. M. H. Veeneklaas, *Folia Haematol.* **65**, 303 (1941).

[42] W. W. Zuelzer and F. N. Ogden, *Proc. Soc. Exptl. Biol. Med.* **61**, 176 (1946).

[43] C. D. May, E. N. Nelson, C. U. Lowe, and R. J. Salmon, *Am. J. Diseases Children* **80**, 191 (1950).

[44] C. D. May, R. D. Sundberg, and F. Schaar, *J. Lab. Clin. Med.* **36**, 963 (1950).

Very low levels of folic acid and folinic acid are found in the livers of the deficient monkeys.[45] The degree of megaloblastosis is proportional to the folinic acid depletion, and those agents which increase the folinic acid level in the liver reverse megaloblastic marrows to normoblastic ones. Folic acid, ascorbic acid, and folic acid conjugate (parenteral) have this property.

In early publications, May and his coworkers felt that ascorbic acid deficiency interferes with the metabolism of folic acid compounds and, in animals on reduced folic acid intake, causes severe deficiency of metabolically active folic acid compounds. The scurvy seems to add a conditioned deficiency of folic acid to a dietary deficiency of this substance. This theory is strengthened by the importance of ascorbic acid to the conversion of folic acid to folinic acid in liver slices, as pointed out by Nichol and Welch,[46] and the greater therapeutic efficiency of folinic acid as compared to folic acid in this monkey anemia.[44] Furthermore the fact that deficiencies of both folic acid and ascorbic acid in guinea pigs, monkeys, and infants induce the same abnormality in tyrosine and phenylalanine metabolism is additional evidence favoring this theory and emphasizing the close chemical relationship of these two compounds. In ascorbic acid-deficient infants, this abnormality in metabolism and excretion is rapidly rectified by ascorbic acid and to some extent by large doses of folic acid.

However, May and his associates believe now that ascorbic acid deficiency merely acts as a "stress factor," possibly through the pituitary-adrenal axis, which places a greater demand on folic acid metabolism than usual.[45] They can find no direct evidence from the folinic acid levels in the livers of their animals that ascorbic acid deficiency interferes with the conversion of folic acid conjugates or folic acid to folinic acid. They conclude that ascorbic acid is not essential for the normal metabolism of folic acid compounds. This more recent theory does not explain the increased levels of folinic acid in the livers of the monkeys treated with ascorbic acid, nor the better response of these animals to folinic acid than to folic acid. It is evident that this chapter is not yet complete. The inference is strong, however, that many cases of megaloblastic anemia in infancy are analogous to this monkey anemia.

In adult persons also, ascorbic acid deficiency provokes many mechanisms through which anemia may occur.[38, 47-51] There may be external blood loss

[45] C. D. May, A. Hamilton, and C. T. Stewart, *Blood* **7**, 978 (1952).

[46] C. A. Nichol and A. D. Welch, *Proc. Soc. Expt. Biol. Med.* **74**, 52 (1950).

[47] S. R. Mettier, G. R. Minot, and W. C. Townsend, *J. Am. Med. Assoc.* **95**, 1089 (1930).

[48] R. B. McMillan and J. C. Inglis, *Brit. Med. J.* **2**, 233 (1944).

[49] B. Gottlieb, *Brit. Med. J.* **2**, 119 (1945).

[50] H. F. Dunlop and H. Scarborough, *Edinburgh Med. J.* **42**, 476 (1935).

[51] L. J. Wade, E. W. Czebrinski, and G. Wood, *J. Missouri State Med. Assoc.* **43**, 756 (1946).

from the gums or from some local lesion like a duodenal ulcer or tissue hematoma. Should this be the predominant etiologic factor, the anemia will be similar morphologically to the anemias of acute and chronic blood loss, that is, normocytic or microcytic hypochromic, and the bone marrow will be hyperplastic and normoblastic. General malnutrition or some other chronic disease such as ulcerative colitis or rheumatoid arthritis associated with scurvy may lead to normocytic normochromic anemia and a normally cellular or mildly fatty hypocellular marrow. None of these types of anemia will respond specifically to administration of ascorbic acid except in so far as this vitamin heals scorbutic lesions and stops bleeding. Anemia does not occur in pure ascorbic acid deficiency of mild degree such as has been observed in human subjects who have submitted themselves to five months or more of ascorbic acid-free diets which are adequate in all other known essential nutrients.[13, 15] Even the loss of 6000 ml. or more of blood during this period is insufficient stress to induce anemia. Patients with mild scurvy seldom have anemia. However, patients with severe scurvy may have one of several types of anemia related directly to the deleterious effect of ascorbic acid deficiency on bone marrow function.

The anemia may be moderate or severe, depending upon the severity of the scurvy. Erythrocyte counts as low as 1.75 million per cubic millimeter have been observed. These cells may be normochromic and normocytic or sometimes slightly macrocytic. White blood cell counts tend to be low, usually under 6000 per cubic millimeter. Differential white blood cell counts are normal, and platelets may be somewhat reduced in numbers. Initial reticulocyte counts are elevated, sometimes as high as 10 to 12%.

In the usual case the bone marrow obtained by sternal aspiration may vary from slight hypercellularity to moderate hypocellularity. Some of the marrow specimens are excessively fatty. There is a relative increase in erythrocyte progenitors, with the majority of these cells at the normoblast and late erythroblast stages of development. Occasionally megaloblasts and bizarre metamyelocytes typical of erythrocyte maturation factor deficiency may be found.[38, 52] This finding is usually the only evidence that one is dealing with a second type of scorbutic anemia.

The severity of the anemia correlates well with the severity of the scurvy, with the elevation of the indirect reacting bilirubin in the blood, and with the reticulocytosis. There is no correlation with the amount of blood loss externally or into the tissues, and serum iron levels are usually normal.

Bed rest alone may be sufficient to induce a remission in the anemia, even though the patient is kept on a diet grossly deficient in ascorbic acid and the vitamins of the B complex. Should no remission occur under

[52] G. H. Jennings and A. J. Glazebrook, *Brit. Med. J.* II, 784 (1938).

these circumstances, then ascorbic acid without any additional factors will induce rapid blood regeneration. In the average case without megaloblastosis the jaundice clears, reticulocytes slowly fall to normal, and the erythrocytes and hemoglobin begin to increase 5 to 8 days after ascorbic acid is begun and reach normal values in about 3 weeks (see Fig. 15). In patients with megaloblastic marrows, administration of ascorbic acid may provoke a reticulocyte response similar to that seen in pernicious anemia when liver

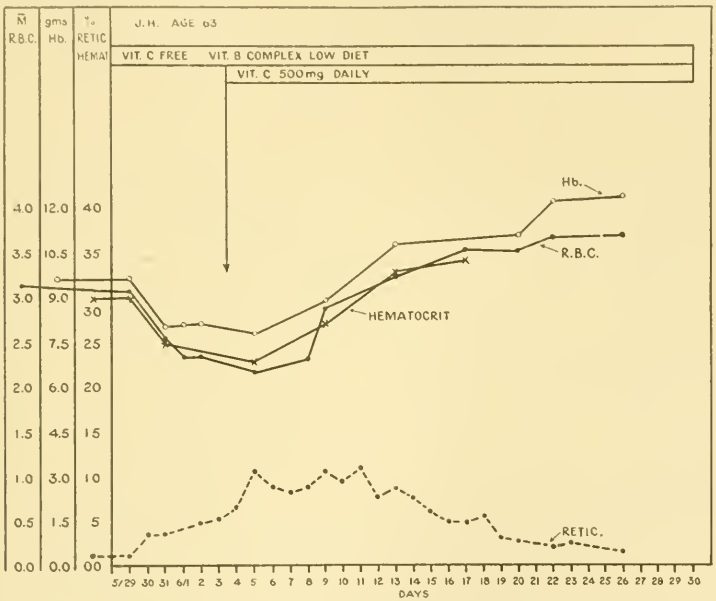

FIG. 15. Hematologic response to ascorbic acid in a patient with severe scurvy. Prior to administration of vitamin C, the anemia was becoming more severe and the reticulocytes were rising, even though the patient was not losing blood externally or developing new ecchymoses. The response to vitamin C alone was rapid.

extract, folic acid, or vitamin B_{12} are given (see Fig. 16). In general, the marrow becomes normoblastic and hyperactive; and if megaloblasts were present, they disappear.

No other substance has been found which will induce such a dramatic therapeutic response in the anemia. Refined liver extract, folic acid and iron have no effect on the anemia, although bizarre metamyelocytes disappear from the marrow after folic or folinic acids are given. Vitamin B_{12} has not had an adequate trial, but it is unlikely that it would have any effect since refined liver extract failed.

The morphologic evidence and therapeutic trials suggest that several mechanisms besides those of blood loss and chronic debilitating illness are

operating to cause anemia. The initial reticulocytosis and the increase in indirect reacting bilirubin out of porportion to the extent of the bleeding into the tissues suggests some mechanism for increased blood destruction or faulty utilization of heme pigments in scurvy. The relatively inactive marrow which becomes hyperactive when ascorbic acid is given suggests that ascorbic acid deficiency suppresses marrow activity in some unknown

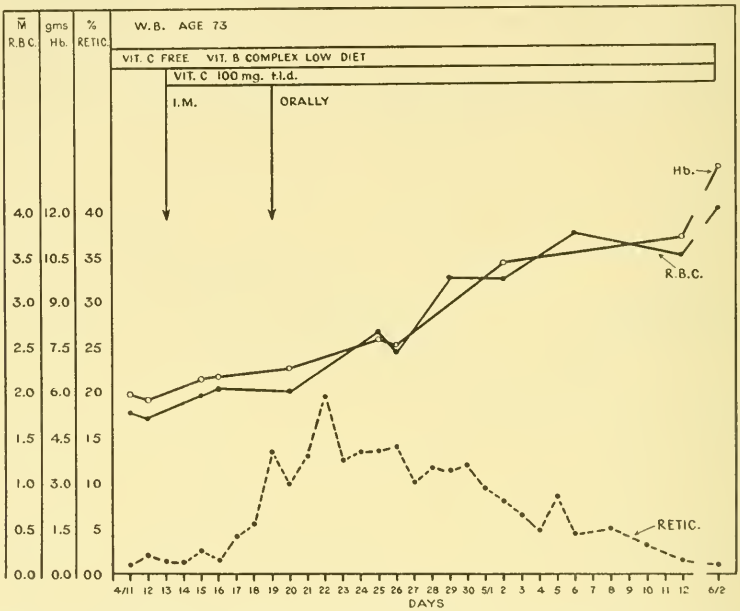

FIG. 16. Hematologic response to ascorbic acid in a patient with severe scurvy and a megaloblastic bone marrow. The reticulocytosis is of the same type that one would expect in a patient with pernicious anemia treated with folic acid or vitamin B_{12}. The ascorbic acid in this patient converted the megaloblastic marrow to a normoblastic one.

fashion. The megaloblastic features seen occasionally suggest that ascorbic acid deficiency has interfered with the metabolism of folic acid in a patient whose folic acid reserves are badly strained by a grossly inadequate diet. Such a mechanism has been postulated to explain the occurrence of megaloblastic anemia of infancy.[43]

However, in scorbutic monkeys, May and his associates[53] have been unable to find any abnormality in protoporphyrin or coproporphyrin and explain the reticulocytosis and increase in urobilinogen on the basis of petechiae and bleeding into the tissues. The scorbutic anemia in their monkeys is hypochromic and the serum iron levels are low, in spite of high

[53] E. C. Prochl and C. D. May, *Blood* 7, 671 (1952).

iron intake, orally or parenterally, a feature which they explain on the basis of poor iron absorption, blood loss, and "stress." This anemia of scurvy in monkeys reproduces the hypochromic anemia seen in scorbutic infants but not the normochromic, normocytic, or slightly macrocytic anemia found in adult persons with scurvy.

It is probable that the anemia of scurvy depends upon the interaction of ascorbic acid deficiency and multiple dietary inadequacies on bone marrow function, yet ascorbic acid alone will restore these failing metabolic reactions. Therefore, the author believes that ascorbic acid has a direct effect on blood formation.

Other indirect effects of ascorbic acid on blood formation have been reported from time to time in the literature. Pernicious anemia and related macrocytic anemias in persons with severe ascorbic acid depletion or frank scurvy may not respond to refined liver extract until ascorbic acid is given. An interesting report from England describes relapses in three persons with pernicious anemia during the winter of 1941 when the English diet was severely lacking in ascorbic acid. Administration of this vitamin restored the effectiveness of the liver extract.[54]

Reticulocytosis and erythrocyte responses have been occasionally reported in persons with pernicious anemia and related macrocytic anemias following treatment with large doses of ascorbic acid.[55] Very low plasma and white blood cell-platelet ascorbic acid levels have been found in 20 unselected patients with pernicious anemia, none of whom had the clinical signs of scurvy.[56] These incidents support the concept of the biochemical interrelationship of ascorbic acid, folic acid, and vitamin B_{12} to which reference has already been made.

Ascorbic acid has no beneficial effect in the anemias of infection, rheumatoid arthritis, iron deficiency, chronic blood loss, or leukemia.

D. PATHOLOGY AND PATHOLOGIC PHYSIOLOGY

The fundamental pathologic change in ascorbic acid deficiency occurs in tissues of mesenchymal origin and is best exemplified in newly formed fibrous tissues.[57-59] The same type of alteration occurs in teeth, growing bone, and blood vessel walls, and accounts for the changes observed in these structures by the clinician.

In connective tissue the fibroblast lies in an amorphous ground substance

[54] S. C. Dyke, B. L. Della Vida, and E. Delikat, *Lancet* **II**, 278 (1942).
[55] R. W. Vilter, *Symposia on Nutrition* **I**, 179 (1947).
[56] R. O. Wallerstein, J. W. Harris, and G. Gabuzda, *Proc. Am. Fed. Clin. Research Abstr.* **119**, (1952).
[57] S. B. Wolbach and O. A. Bessey, *Physiol. Revs.* **22**, 233 (1942).
[58] S. B. Wolbach, *J. Am. Med. Assoc.* **108**, 7 (1937).
[59] G. Dalldorf, The Vitamins, p. 339. American Medical Association, 637 pp., 1939.

within which fibrils are formed. The fibrils, cemented together by a translucent matrix, presumably a product of the fibroblast, are converted into wavy bands of collagen. In ascorbic acid deficiency, the matrix or ground substance is defective and normal collagen is not formed. For this reason the tensile strength of fibrous tissue in healing wounds is poor and the wounds tend to open. Studies with hyaluronidase and ribonuclease suggest that this matrix or ground substance which is defective in ascorbic acid deficiency is a mucopolysaccharide.[60]

In growing bone, particularly in the bones of infants, the bone matrix, ground substance, or osteoid is defective also. In order to understand the pathology which ensues, one must be able to visualize clearly the normal histology of endochondral ossification. Two areas of ossification appear in the cartilage at each end of the developing long bone; one is within the epiphysis and the other at the end of the diaphysis. The latter is of most importance to this discussion. The cartilage cells multiply, line up in columns, and those nearest to the shaft of the bone swell, become vacuolated, and begin to degenerate. The cartilaginous matrix around these columns is calcified and becomes the zone of provisional calcification which bridges and strengthens the gap between hyaline cartilage and spongy bone. Blood vessels and connective tissue from the bone marrow of the shaft invade this area of provisional calcification, penetrate the cartilage columns and destroy the cartilage cells. Specialized fibroblasts in this connective tissue are the precursors of the osteoblasts that finally line the walls of the columns. The interstitial calcified substance derived from cartilage between the columns is dissolved, and new matrix is laid down by the osteoblasts which is rapidly ossified, forming the trabeculae of new bone. The epiphyseal center of ossification progresses in much the same fashion along the articular boundary.

In scurvy, calcification is not affected and the zone of provisional calcification forms normally or is even more dense than usual because the growth of cartilage slows down and the columns of cartilage cells become irregular and compressed. Osteoblasts which were invading this area of provisional calcification change histologically and take on the appearance of fibroblasts as they migrate back shaftward. What matrix they produce does not seem to gel and appears more like loose connective tissue. It does not ossify because it is abnormal matrix. Therefore, one has at the epiphyseal end of the diaphysis a dense zone of provisional calcification, underneath which there are irregular masses of calcified cartilage in fibrous tissue poor in collagen. Until growth ceases entirely these areas are pushed shaftward and piled up in an irregular fashion in an area of the shaft usually ossified and occupied by marrow tissue. Still farther shaftward is loose

[60] J. R. Penney and B. M. Balfour, *J. Pathol. Bacteriol.* **61**, 171 (1941).

connective tissue with few cells and much ungelled matrix. This zone, free of trabeculae, is the roentgenologist's zone of rarefaction. The whole irregular mass is the scorbutic lattice. The area of fibrous tissue in which the irregular masses of cartilage are embedded in helter-skelter fashion has been called the "Trümmerfeld zone" (a field full of debris) and the "Gerüstmark" (framework) in the German literature.

Though ossification is slowed down or halted in the abnormal matrix, resorption of bone continues, and the shaft and ossified center in the epiphysis become thin and porous.

These changes account for the x-ray pattern in the bones of scorbutic infants. The white line of scurvy is the piled-up zone of provisional calcification. The "halo" epiphysis is due to a similar white line surrounding the epiphyseal zone of ossification with reabsorption of bone toward the epiphyseal center. The radiolucent zone under the white line is the zone of rarefaction through which nicking of the cortex and eventually fractures and displacement of the epiphyseal end of the bone may occur.

In general, the same abnormalities occur in the growing flat bones but they are not so conspicuous except at the costochondral junctions.

Periosteal growth continues but in an abnormal fashion. A layer of cells without intercellular material forms and separates periosteum from bone. The subperiosteal hemorrhages begin at sites of fracture through the zone of rarefaction and spread into this friable tissue, stripping the periosteum back along the shaft and to, but not beyond, the epiphyses.

In the teeth, growth of dentine ceases, the pulp becomes separated from the dentine by liquid derived from odontoblasts. The dentine becomes porous. Resorption of alveolar bone occurs, but new bone formation does not take place. The teeth, therefore, become loose and fall out.

It is presumed but not proved that similar changes occur in the capillary walls. Intercellular cement substance is lost, and capillary fragility and permeability increase, leading to the petechiae, ecchymoses, and hematomata. Rumpel-Leede and similar tests become positive. The gingival margins of persons with teeth are supplied by "end capillaries" which cannot anastamose freely with adjacent capillary beds. When hemorrhages occur in these vessels, thromboses and infarctions follow. The gums become swollen, blue-red, and boggy with blood. Infection, so common around poorly cared-for teeth of nutritionally deficient patients, spreads rapidly; the mouth becomes foul and painful, and the gums infarcted and necrotic. Alveolar bone is resorbed and patients frequently perform autoextraction. Gum lesions never occur in the mouths of edentulous patients.

Striated muscle is fragmented, and there is multiplication of sarcolemma nuclei. This is not a specific pathologic lesion for it occurs in many diverse diseases. Hemorrhage into muscle is common.

No abnormalities except occasional small hemorrhages have been found in the adrenal glands of human beings with scurvy even though there appears to be some relationship between ascorbic acid and adrenal function.[61-65] Such a relationship is suggested by the lowered ascorbic acid level of the adrenal gland under stress or treatment with adrenocorticotropic hormone, the lowered cholesterol of the adrenal gland under these circumstances, and the decrease in the urinary output of cortical steroids by scorbutic guinea pigs. However, tests of adrenal function in monkeys[66] and human beings[67] with scurvy so far have been normal, and recent estimations of the output of 17-ketosteroids and formaldehydegenic substances in the urine of scorbutic guinea pigs give no indication of adrenal insufficiency.[68, 69]

The connection between the physiologic and biochemical abnormalities which are now known to occur in ascorbic acid-deficient animals and human beings and the pathology observed in these subjects is not clear. As already mentioned, ascorbic acid is necessary for the complete metabolism of tyrosine, phenylalanine, and dihydroxyphenylalanine (dopa). Errors in the metabolism of these amino acids and the interference with the metabolism of dihydroxyphenylalanine may account for the excessive pigmentation of the scorbutic patient. These metabolic abnormalities result in a type of alkaptonuria sometimes called "tyrosyluria."

The abnormality in folic acid metabolism induced by ascorbic acid deficiency may account for the macrocytic type of scorbutic anemia as it may account for some cases of megaloblastic anemia of infancy. Abnormalities in the metabolism of mucopolysaccharides, possibly mediated through abnormalities in enzyme systems such as the hyaluronidases, may account for the lesions of bone, blood vessels, and fibrous tissues.

It is evident from the many morphologic lesions of scurvy and the diverse biochemical reactions known to be affected adversely by a deficiency of ascorbic acid that this vitamin is of very wide physiologic importance. The fact that it is such a strong reducing agent has led to the suggestion that it acts as a regulator of oxidation-reduction potential throughout the

[61] G. Sayers, M. A. Sayers, H. L. Lewis, and C. N. H. Long, *Proc. Soc. Exptl. Biol. Med.* **55**, 238 (1944).

[62] M. A. Sayers, G. Sayers, and L. A. Woodbury, *Endocrinology* **42**, 379 (1948).

[63] W. H. Daughaday, H. Jaffe, and R. H. Williams, *J. Clin. Endocrinol.* **8**, 244 (1948).

[64] J. C. Beck, M. M. English, J. W. Hackney, and K. R. Mackenzie, *J. Clin. Invest.* **29**, 798 (1950).

[65] Editorials and Comments: *J. Am. Med. Assoc.* p. 825 (1951).

[66] C. T. Stewart, R. J. Salmon, and C. D. May, *J. Lab. Clin. Med.* **40**, 657 (1952).

[67] H. S. Treager, G. J. Gabuzda, N. Zamcheck, and C. S. Davidson, *Proc. Soc. Exptl. Biol. Med.* **75**, 517 (1950).

[68] B. E. Clayton and F. T. Prunty, *Brit. Med. J.* **II**, 927–930 (1951).

[69] E. M. Nadel and J. J. Schneider, *Federation Proc.* **11**, 263 (1952).

body and is therefore essential to many different chemical reactions and enzyme systems. It seems probable that this last hypothesis will eventually prove to be correct, but for the present we cannot describe the clinical lesions of ascorbic acid deficiency in terms of abnormalities in specific chemical reactions.

E. DIFFERENTIAL DIAGNOSIS

Ascorbic acid deficiency, scurvy, must be differentiated from rickets in infancy. In fact the two diseases commonly occur together and may produce a confusing picture in long-bone x-ray studies. Under these circumstances the roentgenologic features of rickets overshadow those of scurvy. Rickets interferes with calcification, osteoid is formed normally, and the epiphyseal ends of the bones are broadened, mushroomed, and radiolucent as opposed to the white line, the Trümmerfeld zone, and the Gerüstmark of scurvy. The rachitic costochondral junction is swollen and smooth, and the scorbutic one is sharp and indented. The white line of scurvy may resemble superficially the heavy epiphyseal line of lead poisoning.

Purpura due to thrombocytopenia, either primary, or secondary to infectious diseases, the ingestion of drugs, or the replacement of marrow by leukemia or neoplasm may be differentiated from scurvy by the low platelet count, the prolonged bleeding time and clot retraction, and the abnormal serum prothrombin concentration even if the purpura simulates perifollicular hemorrhages. Toxic, embolic, vascular, or allergic purpuras due to drugs, bacterial endocarditis, glomerulonephritis, rheumatic fever, or related diseases such as Henoch-Schoenlein's purpura and disseminated lupus erythematosis, all of which may have superficial similarities to scurvy, may be differentiated from scurvy by finding other signs of the causative disease such as positive blood culture, heart murmurs, high fever, or much blood and other formed elements in the urine. It should always be remembered that scurvy will probably not be manifest if a patient has eaten as much as one or two oranges in the last one or two months.

The gum lesions of scurvy are mimicked only by dilantin toxicity, and this should be remembered when an epileptic person is found to have scurvy-like gum lesions. Ordinary inflammatory gingivitis is red, scorbutic gingivitis is a bluish-red color, and the gums are much more friable. Gingivitis due to ascorbic acid deficiency seldom occurs without other florid manifestations of scurvy.[70]

The muscle pains and swollen joints of some patients with scurvy may suggest rheumatoid arthritis, but patients with arthritis will not usually have bloody joint effusions, or other signs of scurvy, and the scorbutic patient will not have deformities such as are common to rheumatoid

[70] J. S. Restarski and M. Pijoan, *J. Am. Dental Assoc.* **31**, 1323 (1944).

arthritis. Subperiosteal hemorrhages in infants may also suggest arthritis, but careful examination will demonstrate that the subperiosteal hemorrhages do not involve the joint.

In children the ecchymoses and joint hemorrhages common to hemophilia may be confused with scurvy. However, the clotting time of the hemophiliac is usually long and the serum prothrombin concentration high.

As has been demonstrated, laboratory tests for ascorbic acid in serum or urine are helpful in eliminating scurvy from consideration if any of this vitamin is found. Saturation tests or tolerance tests indicate only saturation or unsaturation of the tissues which may exist for months before scurvy appears. A level of 0 mg. % in the white cell-platelet layer of the buffy coat is the only laboratory evidence of scurvy or the immediate prescorbutic state.

X. Pharmacology

RICHARD W. VILTER

Ascorbic acid is a white, odorless crystalline substance, molecular weight 176.06. In the dry state it is reasonably stable in air, but it rapidly deteriorates in aqueous solution in the presence of air. It is heat labile. One gram is soluble in 3 ml. of water, 25 ml. of alcohol, 50 ml. of absolute alcohol, or 100 ml. of glycerin. It is insoluble in benzene, chloroform, ether, petroleum ether, or fat. The L isomer is the physiologically active form. Reduced ascorbic acid comes to equilibrium with its oxidation product, dehydroascorbic acid, also antiscorbutic, which is converted above pH 4 to 2,3-diketogluconic acid and thence to oxalic and L-threonic acids. Either ascorbic acid or its sodium salt may be given orally or parenterally. The sodium salt is preferable for intramuscular injection because it causes less local pain than the acid substance. Ascorbic acid is available in 15-, 25-, 50-, 100-, and 250-mg. tablets for oral use and as the sodium salt in 100-, 250-, 500-, and 1000-mg. ampules for intravenous or intramuscular use. Within very wide limits of dosage there are no toxic effects, although, as with any chemical compound which is given intravenously, very occasional anaphylactoid reactions may occur.

After either oral or parenteral administration, ascorbic acid is excreted rapidly in the urine of persons whose tissues are saturated with it. It appears in the urine most rapidly after being given by the intravenous route. It is excreted by the glomeruli and resorbed by the tubules. T_m for the tubules is reported to be 1.2 to 2.1 mg. per 100 ml. of glomerular filtrate (the renal

threshold is 1.4 mg. %)[1, 2] This threshold falls as the state of deficiency progresses. There is always some vitamin cleared even when the tissues are severely depleted. Under these circumstances less than 5% of a test intravenous dose of 100 mg. will appear in the urine in 3 hours, whereas, when this dose is given to a person whose tissues are saturated, more than 50% will appear in the urine in 3 hours.

Ascorbic acid also appears in the stool, but not more than 6 to 10 mg. is excreted by this route in 24 hours even when large doses are fed.[3] Diarrhea increases excretion by the fecal route.

Ascorbic acid is found in greatest concentration in tissues of high metabolic activity. It is highest in the retina, and occurs in decreasing concentration in the following tissues; pituitary gland, corpus luteum, adrenal cortex, young thymus, liver, brain, testes, ovaries, spleen, thyroid, pancreas, salivary glands, lungs, kidney, intestinal wall, heart muscle, spinal fluid, white blood cells, erythrocytes, and plasma.[4]

Physical stress such as occurs during surgical operations or following severe burns or shock of any type induces a precipitous fall in the plasma ascorbic acid level and in the amount excreted in the urine with or without a load or tolerance test.[5, 6] Massive doses of ascorbic acid given orally or parenterally can restore the plasma level temporarily to normal. Normal levels can be maintained only by high dosage with the vitamin until the stress is terminated. Ascorbic acid seems to be diverted from the plasma and urine to the tissue or to storage depots. Many other plasma constituents including iron and the B complex vitamin react in the same way during stress, and the fact that plasma levels are low does not necessarily indicate that there is a deficiency of the substance, nor that more of the substance is being utilized and therefore is required in much larger amounts than usual. In the case of ascorbic acid such low levels do not mean that wounds will not heal unless high plasma levels are maintained by massive dosage, although the surgical literature implies that such is the case. Serum iron levels fall when the adrenals are stimulated by ACTH but so far this parallelism has not been demonstrated for ascorbic acid and much more investigation is needed before the function of ascorbic acid during stress will be clarified.

[1] E. P. Ralli and S. Sherry, *Medicine* **20**, 251 (1941).
[2] J. M. Faulkner and F. H. L. Taylor, *J. Clin. Invest.* **17**, 69 (1938).
[3] A. F. Abt and C. J. Farmer, The Vitamins, p. 411. American Medical Association, 1939.
[4] M. Yavorsky, P. Almaden, and C. G. King, *J. Biol. Chem.* **106**, 525 (1934).
[5] C. C. Lund, S. M. Levenson, R. W. Green, R. W. Paige, P. E. Robinson, M. A. Adams, A. H. McDonald, F. H. L. Taylor, and R. E. Johnson, *Arch. Surg.* **55**, 557 (1947).
[6] C. C. Lund and J. H. Crandon, *J. Am. Med. Assoc.* **116**, 663 (1941).

A dose of 50 to 100 mg. will clear lesions of scurvy, although it may take 4 g. of the material to saturate the tissues of scorbutic subjects. The usual dose in persons suspected of scurvy is 100 mg. three or five times a day by mouth, continued until 4 g. have been administered, then 100 mg. twice a day. In patients who are critically ill with scurvy 1000 mg. can be given daily by intravenous drip or in divided doses of 100 mg. each intramuscularly. In infants and young children, a dose of 10 to 25 mg. three times a day will be adequate.

Scorbutic lesions heal rapidly when vitamin C is given. Apathy, listlessness, severe weakness, and shock-like states disappear and periodic breathing is normalized within 24 hours. Spontaneous bleeding ceases within the same period of time. Fever disappears within 24 to 48 hours as does bone and muscle pain. Within 2 to 3 days the gums lose their blue-red appearance and begin to heal. Perifollicular hemorrhages turn brown in 3 to 4 days and, in healing, leave a pigmented hyperkeratotic papule around the follicle. Even large ecchymoses and deep hematomata are resorbed in 10 to 12 days. The skin may show pigmentation for months, particularly in the areas of extensive hemorrhages.

Blood regeneration begins almost immediately. In 3 to 5 days the marrow is hyperactive and normoblastic. Erythrocyte and hemoglobin levels will increase in 5 to 7 days and will reach normal in 2 to 4 weeks, depending on the severity of the anemia. If complications such as chronic infection, severe renal disease, or cachexia are present, blood regeneration may be slow.

Serum bilirubin and urine and stool urobilinogen levels are within normal limits 3 to 5 days after ascorbic acid is begun. The excessive excretion of "tyrosyl" substances in the urine after a test dose of tyrosine is normalized within 48 hours.

Ascorbic acid has been suggested, without confirmatory evidence, for the treatment of numerous and diverse diseases unrelated to a deficiency of this substance, principally because it is a strong reducing agent and is alleged to have potent detoxifying properties. For these reasons it has been used in the treatment of hay fever and asthma[7] and in conjunction with histidine in peripheral arterial disease.[8] It has been combined with mercurial diuretics because it is supposed to reduce toxicity, and it has been used in other heavy metal intoxications for the same reason.[9] It has been recommended in the treatment of barbiturate poisoning. It has been used in the treatment of congenital methemoglobinemia with reduction in the

[7] S. L. Ruskin, *Am. J. Digest. Diseases* **12**, 281 (1945).

[8] M. T. Friedell, E. F. Drucker, and W. J. Pickett, *J. Am. Med. Assoc.* **138**, 1036 (1948).

[9] H. N. Bundesen, H. C. S. Aron, R. S. Greenebaum, C. J. Farmer, and A. F. Abt, *J. Am. Med. Assoc.* **117**, 1692 (1941).

methemoglobin, improvement in the color of the patient, and relief of the secondary polycythemia while administration is continued, but with no lasting beneficial effects.[10-13] It is alleged to have antihemolytic effects also.[14]

It has been claimed that ascorbic acid has anti-infection properties and an effect on complement formation.[15, 16] There is no doubt that ascorbic acid levels in the plasma are low and that urinary excretion is reduced[17, 18] in infectious diseases, especially tuberculosis, and that much larger doses are required to keep these levels in the normal range. This does not mean, however, that ascorbic acid has anti-infection properties. Ascorbic acid has been used as a panacea for bleeding gums[19-22] and, with vitamin K, for bleeding of any type. It has been recommended for colds, brucellosis, tuberculosis, ulcerative colitis, regional ileitis, glomerulonephritis, pertussis, smallpox, schizophrenia, cobra venom poisoning, infectious hepatitis, tonsillitis, and many other unrelated conditions. With the years, this therapeutic enthusiasm has waned, since in none of these conditions did the therapy withstand the test of time.

There have been reports that ascorbic acid increases the absorption of iron,[23] and both the suggestion[24] and the denial[25, 26] that ascorbic acid influences the utilization of iron. Therefore, this vitamin has been included in many iron preparations for oral use. Recent evidence obtained by feeding foods containing iron tagged with isotopic Fe^{59} to animals and human subjects indicates that the absorption of organic food iron is greatly facilitated if reducing substances are administered orally at the same time. Ascorbic acid is the most active of these and increases absorption of organic food iron in normal subjects and those with hypochromic anemia

[10] H. Barcroft, Q. H. Gibson, D. C. Harrison, and J. McMurray, *Clin. Science* **5**, 145 (1945).
[11] R. F. Sievers and J. B. Ryon, *Arch. Internal. Med.* **76**, 299 (1945).
[12] M. Carnrick, B. D. Polis, and T. Klein, *Arch. Internal Med.* **78**, 296 (1946).
[13] C. A. Finch, *Bull. New Engl. Med. Center* **9**, 241 (1947).
[14] V. Traina, *Nature* **164**, 843 (1949).
[15] E. E. Ecker, L. Pillemer, D. Wertheimer, and H. Gradis, *J. Immunol.* **34**, 19 (1938).
[16] E. E. Ecker, L. Pillemer, J. J. Griffitts, and W. P. Schwartz, *J. Am. Med. Assoc.* **112**, 1449 (1939).
[17] J. M. Faulkner and F. H. L. Taylor, *Ann. Internal Med.* **10**, 1867 (1937).
[18] M. A. Abbasy, L. J. Harris, and P. Ellman, *Lancet* **II**, 181 (1937).
[19] F. S. Roff and A. J. Glazebrook, *J. Roy. Navy Med. Serv.* **25**, 340 (1939).
[20] F. Stuhl, *Lancet* **I**, 640 (1943).
[21] B. S. Kent, *Lancet* **I**, 642 (1943).
[22] M. T. Hanke, *J. Am. Dent. Assoc.* **17**, 957 (1930).
[23] H. Schröder and M. Braun-Stappenbeck, *Klin. Wochschr.* **20**, 979 (1941).
[24] H. Albers, *Arch. Gynäkol.* **172**, 547 (1942).
[25] A. Videback and G. Alsted, *Acta Med. Scand.* **114**, 403 (1943).
[26] H. V. Schulze and A. F. Morgan, *Am. J. Diseases Children* **71**, 593 (1946).

from a level of 3 to 5% to one of 20 to 75%.[27] These data do not justify mixing ascorbic acid with inorganic ferrous salts for oral use, since absorption of pure ferrous iron is so excellent.

Because plasma ascorbic acid levels are low in rheumatoid arthritis[28] and in rheumatic fever,[29] this vitamin has been used for many years in the treatment of these diseases without any definite proof of effectiveness. Recently it has been recommended again, this time with desoxycorticosterone in rheumatoid arthritis, in an attempt to gain a relatively inexpensive cortisone effect.[30] There is no proof that this combination has any beneficial effect.[31] So far, scurvy and subclinical ascorbic acid deficiency are the only conditions affecting human beings in which ascorbic acid has a specific therapeutic effect. Prevention of scurvy, the megaloblastic anemia of infancy, and possibly iron deficiency anemia are the only known prophylactic effects.

XI. Requirements

A. OF ANIMALS

MARY ELIZABETH REID

1. Criteria for Judging Requirements

The vitamin C requirements of the guinea pig have been reviewed by Mannering.[1] As he points out, the requirements should be viewed in relation to the function under consideration. The requirements may differ for growth, for maintenance of adult animals at different ages, and for reproduction, pregnancy, and lactation. Other factors such as temperature, rate and mode of administration of the vitamin, type of diet, and infection and trauma may also influence the need. Various macroscopic and microscopic characteristics may be used in evaluating the requirements. Macroscopic measurements of the vitamin C status may be made by determining the increase in weight of the whole animal, the growth of certain organs such as the teeth and/or adrenals, the blood levels with respect to plasma or white cell concentration of the vitamin, and the level of alkaline serum phosphatase. Microscopic measurements are based on changes in the teeth

[27] C. V. Moore and R. Dubach, *Trans. Assoc. Am. Physicians* **64**, 245 (1951).

[28] J. F. Rinehart, L. D. Greenberg, and F. Baker, *Proc. Soc. Exptl. Biol. Med.* **35**, 347 (1936).

[29] J. F. Rinehart, L. D. Greenberg, and A. U. Christie, *Proc. Soc. Exptl. Biol. Med.* **35**, 350 (1936).

[30] E. Lewin and E. Wassen, *Lancet* **II**, 993 (1949).

[31] J. H. Kellgren, *Lancet* **II**, 1108 (1949).

[1] G. J. Mannering, *Vitamins and Hormones* **7**, 201 (1949).

as shown by the rate of growth of dentine, growth in length of the odontoblasts, and phagocytic activity and fragility.

a. Prevention of Gross Symptoms of Scurvy

A variety of scorbutigenic diets, in many of which autoclaved alfalfa has been incorporated, have been successfully employed for determining the requirements. Aged rabbit chow pellets (commerical) containing alfalfa have also been so used. In recent years, however, there has been considerable difficulty in producing scurvy in the expected time with these pellets, even when they are heated to destroy the ascorbic acid. The generally accepted explanation for this difference is that the alfalfa presently used is a superior product and is dried under conditions which tend to preserve the ascorbic acid. Because of these difficulties some workers in this field are now using a modified form of the Sherman, LaMer, and Campbell[2] diet. The writer has found the following modifications to be satisfactory;

	g./kg.		g./kg.
Rolled oats	400	NaCl	10
Wheat bran	200	$CaCO_3$	20
Casein (vitamin-free)	289	Brewer's yeast (dried)	1
Butter fat	40	Fat-soluble vitamins	
Corn or cottonseed oil	40		

The following fat-soluble vitamins are added to the diet after dissolving in the oil using the procedure followed by Booth *et al.*[2a]

β-Carotene	5 mg.
Calciferol	1 mg.
Vitamin E acetate	10 mg.
Vitamin K	2 mg.

Sherman *et al.*[2] showed that gross protection against scurvy was afforded by 3 ml. of tomato juice, a quantity which presumably contained about 0.5 mg. of ascorbic acid. In tests with synthetic ascorbic acid other investigators have found a daily dosage of approximately 0.5 mg. to be sufficient[3-11] for the prevention of gross symptoms of scurvy.

[2] H. C. Sherman, V. K. LaMer, and H. L. Campbell, *J. Am. Chem. Soc.* **44**, 165 (1922).

[2a] A. N. Booth, C. A. Elvehjem, and E. B. Hart, *J. Nutrition* **37**, 263 (1949).

[3] W. A. Waugh and C. G. King, *J. Biol. Chem.* **97**, 325 (1932).

[4] E. W. Crampton, B. C. Collier, L. D. Woolsey, and F. A. Farmer, *Science* **100**, 599 (1944).

[5] C. F. Dunker, C. R. Fellers, and W. B. Esselen, *Food Research* **7**, 260 (1942).

[6] J. L. Svirbely and A. Szent-Györgyi, *Biochem. J.* **27**, 279 (1933).

[7] L. Randoin, *Compt. rend. soc. biol.* **116**, 4 (1934).

[8] G. F. Göthlin, *Nature* **134**, 569 (1934).

[9] A. Giroud, C. P. Leblond, and R. Ratsimamanga, *Yale J. Biol. Med.* **9**, 573 (1937).

[10] A. Djabri, *Z. Vitaminforsch.* **10**, 210 (1940).

[11] E. W. Crampton, *J. Nutrition* **33**, 491 (1947).

b. Growth Measurements

(1) *Increase in Weight of Whole Animal.* An approximately normal growth is obtained in young animals with a daily intake of 0.5 mg. of the vitamin.

(2) *Growth of Certain Organs or Tissues, e.g., Teeth.* (a) Macroscopic Evidence. Dalldorf and Zall[12] found that the rate of growth of the teeth is proportional to the vitamin C intake. The rate of tooth growth was determined by clipping off the exposed portions of the lower incisors at periods varying from 20 to 90 days. The method was recommended as being more accurate than the gross symptom procedure and much simpler than the microscopic technique of Höjer.[13] The rate of dentine deposition in animals given 5 mg. of ascorbic acid daily coincided with that of animals given greens and was considered normal.

(b) Microscopic Evidence. The effect of lack of vitamin C is shown by early changes in the structure of the teeth. Variations occur in shape and arrangement of the odontoblasts and their products, dentine and predentine.

The rate of growth of dentine may also be used in determining the minimum protective dose. By staining the dentine with periodic injections of alizarin the rate of dentine deposition was studied in relation to the intake of vitamin C.[14] After a depletion period of 7 days the amount of dentine deposited in the formative end of the tooth was found to vary directly with the vitamin C intake. The rate of deposition of dentine in guinea pigs given 5 mg. of ascorbic acid coincided with that of animals given greens and was considered as normal.

Measurement of the rate of growth of the odontoblasts has proved to be an especially accurate method. Crampton *et al.*[4, 11] found that a considerably smaller number of animals was required to obtain significant differences between experimental groups than with the "increase in weight" method. A 2-mg. daily dosage was shown by this method to give protection.

The tooth shows a close relation to the level of vitamin C intake.[13, 15-17] The advantages of the microscopic tooth methods for determining the minimum protective dosage are their specificity and the shortness of the test period. Because the microscopic changes in the teeth occur much earlier in the deficient animals than do the macroscopic symptoms, twice as much (presumably 1 mg.) antiscorbutic substance was found to be required by the microscopic tooth method.[18, 19] Later, other workers reported a still

[12] G. Dalldorf and C. Zall, *J. Exptl. Med.* **52**, 57 (1930).
[13] J. A. Höjer, *Acta Paediat. Suppl.* **3**, 8 (1924).
[14] P. E. Boyle, O. A. Bessey, and P. R. Howe, *Arch. Pathol.* **30**, 90 (1940).
[15] S. S. Zilva and F. M. Wells, *Proc. Roy. Soc.* (*London*) **B90**, 505 (1919).
[16] S. B. Wolbach and P. R. Howe, *Arch. Pathol.* **1**, 1 (1926).
[17] L. Jackson and J. J. Moore, *J. Infectious Diseases* **19**, 478 (1916).
[18] M. Goettsch, *Quart. J. Pharm.* **1**, 168 (1928).
[19] W. H. Eddy, *Am. J. Public Health* **19**, 1309 (1929).

higher requirement, namely 2 mg. per day.[20, 21] Kuether *et al.*[22] obtained a value of 3 mg. for 300-g. animals. Values of 2.4 mg.[23] and 2.5 mg.[24] have also been reported. It would thus appear that the minimum requirement as judged by the tooth method is 2 to 3 mg. per day, which is actually four to six times as great as that for the prevention of macroscopic symptoms in other organs and tissues.

c. Twenty-Four-Hour Urinary Excretion of the Vitamin

This method has been found to be an unreliable indicator of the ascorbic acid status[25] in man and would not be usable in the guinea pig, since it does not excrete ascorbic acid except with dosage levels which are higher than the requirements. By the use of test or saturation doses[26] in man, the results are somewhat better, the amount excreted depending on the plasma concentration which in turn depends on the degree of depletion of the tissues.

d. Methods Involving Measurements in Blood

(1) *Fasting Plasma Concentration.* This method has been found to give a more accurate index of the ascorbic acid status than the 24-hour urinary excretion test and is simpler than the test dose procedure.[27, 28] In the guinea pig Giroud *et al.*[9] reported that doses of more than 50 mg. daily were necessary to maintain growing animals in health. With this dosage the amount found in the tissues is about the same as that found in animals which synthesize the vitamin. Most workers in this field, however, consider this level to be higher than is necessary. Penney and Zilva[29] found that an intake of 25 mg. daily was necessary to maintain saturation of the tissues in 300-g. guinea pigs. There appeared to be no definite correlation between the tissue content and the onset of scorbutic lesions, provided that the intake exceeded 1 mg. per day. The survival time of initially saturated animals on a scorbutigenic diet was no longer than that of animals whose initial tissue concentration was about half the saturation value. They interpreted this as suggesting that at least the fraction of ascorbic acid lost from the tissues

[20] L. J. Harris and S. N. Ray, *Biochem. J.* **27**, 2016 (1933).
[21] K. M. Key and B. G. E. Morgan, *Biochem. J.* **27**, 1030 (1933).
[22] C. A. Kuether, I. R. Telford, and J. H. Roe, *J. Nutrition* **28**, 347 (1944).
[23] H. C. Hou, *Chinese J. Physiol.* **10**, 213 (1936).
[24] M. B. Cohen, *Am. J. Diseases Children* **60**, 636 (1940).
[25] E. P. Ralli, G. J. Friedman, and S. Sherry, *J. Clin. Invest.* **18**, 705 (1939).
[26] E. P. Ralli, G. J. Friedman, and M. Kaslow, *Proc. Soc. Exptl. Biol. Med.* **36**, 52 (1937).
[27] L. D. Greenberg, J. F. Rinehart, and N. M. Phatak, *Proc. Soc. Exptl. Biol. Med.* **35**, 135 (1936).
[28] A. F. Abt and C. J. Farmer, *J. Am. Med. Assoc.* **111**, 1555 (1938).
[29] J. R. Penney and S. S. Zilva, *Biochem. J.* **40**, 695 (1946).

in the early stages of depletion does not serve to protect the animal against scurvy. Mannering[1] states that "in view of the minimal ascorbic acid levels found necessary for protection against scurvy, for reproduction, and for prolonged survival, there is little to support the concept that saturation with ascorbic acid is a prerequisite of adequate nutrition."

(2) *Alkaline Serum Phosphatase.* Determination of the content and activity of this enzyme has been suggested for evaluating the nutritional status with respect to ascorbic acid. Gould and Schwachman[30] showed that the normal phosphatase content of guinea pig serum can be maintained by a daily dose of only 0.225 mg. of the vitamin, suggesting a high degree of sensitivity.

(3) *Content in White Blood Cells.* Butler and Cushman[31] stated that the level of ascorbic acid in the white cell-platelet layer in centrifuged blood samples of human subjects constitutes the most accurate index of vitamin C deficiency. The concentration in this layer may lie within the normal range of 25 to 38 mg. per 100 g. of white blood cells, even with a low concentration in the plasma. Crandon *et al.*[32] found that the zero level in the plasma of a human subject kept on a diet lacking ascorbic acid was reached by the forty-first day whereas in the white cell layer the zero level was not reached until the one hundred and twenty-fourth day. The leucocyte layer was the last to become depleted and the first to become saturated when ascorbic acid was supplied. Lowry *et al.*[33] studied the white cell and serum vitamin C levels in three groups of normal young men maintained for a period of 8 months on 8, 23, and 78 mg. of ascorbic acid per day, respectively. When saturation tests were performed the increase in white cell ascorbic acid concentration paralleled its retention. For this reason they stated that the white cells "appear to be a valid index of the total body concentration of ascorbic acid." Chevillard and Hamon[34] made a similar study with guinea pigs and found that in animals deprived of ascorbic acid the leucocyte-platelet layer showed decreases in the vitamin which closely followed those of the body tissues. They found that the ascorbic acid value of the leucocyte-platelet layer was about 30 mg. per 100 g. Values of less than 20 mg. per 100 g. indicated advanced avitaminosis C.

(4) *Phagocytic Activity and Fragility of Polymorphonuclear Leucocytes.* A possible method for determining the requirement for combating infections is suggested in the methods employed by Nungester and Ames.[35]

[30] B. S. Gould and H. Schwachman, *J. Biol. Chem.* **151**, 439 (1943).
[31] A. M. Butler and M. Cushman, *J. Clin. Invest.* **19**, 459 (1940).
[32] J. H. Crandon, C. C. Lund, and D. B. Dill, *New Engl. J. Med.* **223**, 353 (1940).
[33] O. H. Lowry, O. A. Bessey, M. J. Brock, and J. A. Lopez, *J. Biol. Chem.* **166**, 111 (1946).
[34] L. Chevillard and F. Hamon, *Compt. rend. soc. biol.* **137**, 307 (1943).
[35] W. J. Nungester and A. M. Ames, *J. Infectious Diseases* **83**, 50 (1948).

e. Weight of the Adrenal Glands

The weight of the adrenal glands has recently been suggested as a criterion of ascorbic acid adequacy for the guinea pig.[36] Large numbers of animals ranging in weight from 190 to 900 g. were maintained on a scorbutigenic diet supplemented with varying amounts of ascorbic acid for a period of 7 weeks. Quantitative data were obtained as to height of the odontoblasts, ascorbic acid content of the white blood cells, plasma, and adrenals, hemoglobin level, weight of the adrenal glands, and water intake. Of all these measurements the weight of the adrenals proved to be the most sensitive criterion of the ascorbic acid requirement. The size of the adrenal increased as the dietary ascorbic acid decreased. As judged by this criterion the daily requirement was found to be 0.65 mg. per 100 g. for animals weighing less than 400 g. and 0.55 mg. for animals weighing more than 500 g. They concluded that the requirement is related to the amount of metabolic tissue, the proportion of such tissue being larger in the young animals. The adrenal weight procedure would be a simple and practicable one to employ if it proved to be reliable. In recent tests, however, the writer has found the adrenals of scorbutic animals to be only slightly enlarged as compared to those of normal controls. It seems possible that some factor in the diet may act in conjunction with variations in available ascorbic acid to affect adrenal size, e.g., pantothenic acid.

At the present time, the general consensus is that measurement of the length of the odontoblasts is the most sensitive and reliable method for determining the ascorbic acid requirement. This discussion has necessarily been limited chiefly to procedures with guinea pigs. Much still remains to be done in determining their nutritional requirements of factors other than vitamin C. Acquisition of this knowledge could conceivably affect certain aspects of the vitamin C picture and thereby improve the methods of determination of the vitamin C requirement.

2. Factors Influencing Vitamin Requirement

a. Mode of Administration

Greater antiscorbutic efficiency with injected than with orally administered doses of ascorbic acid has been observed in guinea pigs by several investigators.[37-42] Hou[39] found that ascorbic acid given by subcutaneous

[36] W. H. Pfander and H. H. Mitchell, *Federation Proc.* **10**, 391 (1951).
[37] D. J. Stephens and E. E. Hawley, *J. Biol. Chem.* **115**, 653 (1936).
[38] A. Grollman and W. M. Firor, *J. Nutrition* **8**, 569 (1934).
[39] H. C. Hou, *Proc. Soc. Exptl. Biol. Med.* **32**, 1391 (1935).
[40] L. DeCaro, *Hoppe-Seyler's Z. physiol. Chem.* **240**, 179 (1936).
[41] S. S. Zilva, *Biochem. J.* **30**, 1419 (1936).
[42] W. Klodt, *Fortschr. Therap.* **12**, 335 (1936); *Chem. Zentr.* 1754 (1936).

injection was twice as effective as that given by mouth. Similarly Klodt[42] found intravenous injections more effective than oral. A 25-mg. dose injected intravenously was found to saturate the tissues whereas the same amount administered orally was insufficient for saturation.[29] However, Burns *et al.*[43] found the total loss of ascorbic acid during the first 24 hours after administration was nearly equal whether the vitamin was given orally or parenterally. The protective dosage was also equal in the two cases.

b. Rate of Administration

Rate of administration of the vitamin has a marked effect on the tissue storage.[29, 44] Bezssonoff and Wolozyn[45] reported that deprivation of the vitamin in guinea pigs for 4 days or more produced an irreversible effect. Young animals given 0.5 mg. per 100 g. of body weight every day by mouth made normal growth, but if given seven times as much every seventh day by injection or eight times as much every fourth day by mouth, growth was stunted. Zilva[46] observed that an intake of 2 mg. per day produced normal growth and well-being, whereas administration of the same total amount every seventh day resulted in retarded growth and a tendency to develop mild scurvy symptoms. Although it has been reported that administration of the vitamin every second or third day has no obvious deterimental effect,[23, 46] it seems doubtful that this procedure would result in a completely healthy condition, particularly if the growth is very rapid. Since guinea pigs in their natural way of life ingest a small amount of the vitamin with every mouthful of food, it would seem that a reasonable approach to this procedure would represent the ideal regime. This suggestion is partially supported by Penney and Zilva's finding[29] that, with oral administration of 25 mg. of the vitamin in one dose, the content in the tissues was less than half that found with the 25 mg. administered in 50 doses at 10-minute intervals. However, this finding would probably hold for many substances of which there is a plethora.

c. Pregnancy and Lactation

As indicated previously, there is increased need of the vitamin during pregnancy and lactation, the increased requirement for which in the guinea pig has not been fully determined. Crampton and Bell[47] found 2 to 5 mg. per day sufficient for reproduction. In humans the requirement during this period is reported to be increased from two to six times.[48, 49]

[43] J. J. Burns, H. B. Burch, and C. G. King, *J. Biol. Chem.* **191**, 501 (1951).
[44] E. Jacobsen, *Skand. Arch. Physiol.* **72**, 259 (1935).
[45] N. Bezssonoff and M. Woloszyn, *Compt. rend. soc. biol.* **130**, 922 (1939).
[46] S. S. Zilva, *Biochem. J.* **35**, 1240 (1941).
[47] E. W. Crampton and J. M. Bell, *Sci. Agr.* **27**, 57 (1947).
[48] F. Widenbauer, *Klin. Wochschr.* **16**, 600 (1937).
[49] A. Bucher, *Münch. Med. Wochschr.* **84**, 734 (1937).

d. Age

Dann and Cowgill[50] reported that age is not a factor in determining the vitamin C requirement of guinea pigs. An intake of 0.5 mg. per 100 g. of body weight gave full protection to animals of various ages and weights. A more efficient utilization of the vitamin by rapidly growing guinea pigs has been suggested as the explanation for their not having a greater requirement in relation to body weight than adult animals.[51] However, Pfander and Mitchell[36] obtained results which suggest a slightly higher requirement in younger animals.

e. Type of Diet

There is little exact information concerning effects of variations in the basal dietary constituents on the ascorbic acid requirement. With diets high in tyrosine content, presumably there would be a somewhat elevated need of the vitamin. In animals which synthesize ascorbic acid, the addition to the diet of compounds such as methionine and chloretone which have a stimulating effect on its synthesis may be important. The lack of such substances in synthetic diets may be one reason why their growth-promoting ability is less than that of diets composed of natural material, although they contain the same amounts of proteins, carbohydrates, fats, vitamins, and minerals as the synthetic diets.

f. Special Factors Such as Flavonoids

Studies have been made of the effects of substances such as rutin on the vitamin C requirements. A sparing action on ascorbic acid has been reported.[52] Other studies have also been made in this field.[53, 54] There is no clear evidence that the guinea pig requires flavonoids in the diet when ample ascorbic acid is present.

g. Infections

The presence of infection may influence the ascorbic acid requirement in two ways. (1) More of the vitamin tends to be consumed as a result of increased leucocytosis[35] and possibly of fever;[55-65] hence the antiscorbutic

[50] M. Dann and G. R. Cowgill, *J. Nutrition* **9**, 507 (1935).
[51] M. E. Reid, *J. Nutrition* **42**, 347 (1950).
[52] E. W. Crampton and L. E. Lloyd, *J. Nutrition* **41**, 487 (1950).
[53] H. Cotereau, M. Gabe, E. Géro, and J. L. Parrot, *Nature* **161**, 557 (1948).
[54] A. M. Ambrose and F. DeEds, *J. Nutrition* **38**, 305 (1949).
[55] F. X. Hausberger and N. Nevenschwander-Lemmer, *Klin. Wochschr.* **18**, 1119 (1939).
[56] I. L. Stutskii, *Klin. Med. (U. S. S. R.)* **15**, 1084 (1937); *Chem. Zentr.* **1**, 4680 (1938).
[57] G. Marotta and G. Calendoli, *Boll. soc. ital. biol. sper.* **19**, 16 (1944).
[58] F. H. Heise and G. J. Martin, *Proc. Soc. Exptl. Biol. Med.* **34**, 642 (1936).
[59] G. Scoz, *Boll. soc. ital. biol. sper.* **11**, 908 (1936).

requirement presumably is increased. (2) It may necessitate the maintenance of a blood level of ascorbic acid sufficiently high to stimulate phagocytic activity. A possible method for determining this type of requirement is suggested by the studies of Nungester and Ames.[35] Under their experimental conditions a serum level of approximately 0.4 mg. % of ascorbic acid was necessary to provide a high degree of phagocytosis (85 to 90 % of the polymorphonuclear cells active) and protection of the cells from rupture. This serum level is approximately double the level necessary to prevent pathological lesions in the incisor teeth.[66, 67] To produce this desirable degree of phagocytic activity a 300-g. guinea pig would require a daily intake of approximately 6 mg. of the vitamin. As previously stated the "growth of the odontoblast method" appears to be the best at present available for determining the antiscorbutic requirement, but there is ample reason for the suggestion that the requirement for good health in the sense of controlling infection may be as much as twice that necessary for the prevention of scurvy. However, further study is required under a variety of conditions before a conclusive evaluation can be made of this angle of the vitamin C problem.

B. OF HUMAN BEINGS

RICHARD W. VILTER

The National Research Council has suggested a vitamin C intake of 75 to 100 mg. per day for optimum human nutrition. This is the amount necessary to maintain tissue saturation and plasma vitamin C levels of 1 mg. % without excessive loss in urine. However, 18 to 25 mg. per day will keep the tissues half saturated and will prevent scurvy.[68-71] The League of Nations Technical Commission on Nutrition (1938) and the Vitamin C Subcommittee of the Accessory Food Factors Committee of the British Medical

[60] M. A. Abbasy, L. J. Harris, and P. Ellman, *Lancet* **II**, 181 (1937).
[61] E. H. J. Warns, *Ned. Tijdschr. Geneesk.* **82**, 4426 (1938).
[62] O. Dobbelstein, *Z. ges. exptl. Med.* **107**, 532 (1940).
[63] K. Daum, K. Boyd, and W. D. Paul, *Proc. Soc. Exptl. Biol. Med.* **40**, 129 (1939).
[64] Falke, *Klin. Wochschr.* **18**, 818 (1939).
[65] S. L. Osborne and C. J. Farmer, *Proc. Soc. Exptl. Biol. Med.* **49**, 575 (1942).
[66] C. C. Lund, L. A. Shaw, and C. K. Drinker, *J. Exptl. Med.* **33**, 231 (1921).
[67] J. H. Roe, C. A. Kuether, and R. G. Zimler, *J. Clin. Invest.* **26**, 355 (1947).
[68] O. H. Lowry, O. A. Bessey, M. J. Brock, and J. A. Lopez, *J. Biol. Chem.* **166**, 111 (1946).
[69] W. P. Stamm, T. F. Macrae, and S. Yudkin, *Brit. Med. J.* **2**, 239 (1944).
[70] V. A. Najjar, L. E. Holt, Jr., and H. M. Royston, *Bull. Johns Hopkins Hosp.* **57**, 315 (1944).
[71] M. Pijoan and E. L. Lozner, *Bull. Johns Hopkins Hosp.* **73**, 303 (1944).

Research Council (1948) recommended 30 mg. daily. In fact, careful experimental work indicates that 10 mg. daily is sufficient to prevent scurvy and maintain good healing properties of tissues for a period of a year, even if plasma ascorbic acid levels are zero and white cell levels almost zero.[72] There is difference of opinion as to whether subtle tissue deterioration occurs at these subsaturation levels. Changes in the gums have been reported by

TABLE XI

Daily Allowances of the National Research Council

	Ascorbic acid, mg.
Man (154 lb., 70 kg.)	
Sedentary	75
Physically active	75
With heavy work	75
Woman (123 lb., 56 kg.)	
Sedentary	70
Moderately active	70
Very active	70
Pregnancy (latter half)	100
Lactation	150
Children up to 12 years	
Under 1 year	30
1–3 years (27 lb., 12 kg.)	35
4–6 years (42 lb., 19 kg.)	50
7–9 years (58 lb., 26 kg.)	60
10–12 years (78 lb., 35 kg.)	75
Children over 12 years	
Girls	
13–15 years (108 lb., 49 kg.)	80
16–20 years (122 lb., 55 kg.)	80
Boys	
13–15 years (108 lb., 49 kg.)	90
16–20 years (141 lb., 64 kg.)	100

some investigators at these subsaturation levels. Most authors feel that the tissues are not damaged morphologically, but it is impossible to say whether chemical damage or interference with enzyme systems occur. When possible, it is best to adhere to the National Research Council recommendations, (Table XI) but under difficult circumstances such as existed in Great Britain during World War II, it is probable that 18 to 25 mg. per day will

[72] H. A. Krebs, R. A. Peters, K. H. Coward, L. W. Mapson, L. G. Parsons, B. S. Platt, J. C. Spence, and J. R. P. O'Brien, Lancet I, 853 (1948).

prevent scurvy and maintain satisfactory health. Data to support these statements are given in Tables XII and XIII.

The level of intake which produces the maximum levels of ascorbic acid in the plasma and minimum loss in the urine is 100 mg. daily.[73] Eighty milligrams daily will saturate tissues and maintain a high serum level.[68]

Many factors increase requirements for ascorbic acid. Recovery from scurvy seems to do this at least temporarily. Instead of 100 mg. per day, which is required to maintain tissue saturation in the average person, at least 200 mg. per day is required in the scorbutic subject after his tissues have been saturated. Pregnancy, lactation, and thyrotoxicosis increase the

TABLE XII

Excretion and Plasma Levels of Ascorbic Acid at Different Levels of Intake

Intake, mg.	Urinary excretion, mg.	Amount retained, mg.	Plasma level, mg. %
50	11	39	0.85
100	20	80	1.12
200	109	91	1.14
350	259	91	1.15

TABLE XIII

Relationship of Intake of Ascorbic Acid to the Amount in the White Blood Cells and Serum

Intake, mg. per day	WBC ascorbic acid, mg. %	Serum ascorbic acid, mg. %
8	11.9	0.18
23	12.9	0.20
78	24.2	0.79

amount of the vitamin utilized each day. Diarrheal states increase the loss of vitamin in the stool, and achlorhydria decreases the amount of vitamin absorbed before it is changed chemically to inactive substances. Rheumatic fever, rheumatoid arthritis, acute and chronic infections, and situations involving physical stress sidetrack the vitamin from the plasma to the tissues or to storage depots. Increased metabolism stimulated by these conditions increases the need for the vitamin but not necessarily by the amount which would be required to restore the plasma levels to normal. Cold atmosphere increases the excretion of ascorbic acid, but this vitamin does not hasten adaptation to cold.[74] In general the same circumstances which in-

[73] E. P. Ralli, G. J. Friedman, and S. Sherry, *Proc. Soc. Exptl. Biol. Med.* **40**, 604 (1939).

[74] N. Glickman, R. W. Keeton, H. H. Mitchell, and M. K. Fahnestock, *Am. J. Physiol.* **146**, 538 (1946).

crease the need for other vitamins, increase the need for ascorbic acid also. These are conditions characterized by increased metabolism, by increased loss of the vitamin in urine, stool, or vomitus, by decreased absorption, or by increased destruction.

In 1757, James Lind[75] wrote almost as clearly on the circumstances surrounding the genesis of ascorbic acid deficiency as we can today. Although he was mistaken as to the bad affects of salt water and salt air, his powers of observation led him to discover the true cause of scurvy and many of the factors influencing its occurrence. He listed the following predisposing factors to scurvy: recovery from other diseases, weakness of the digestive tract, indolence, mental disturbances (as in impressed seamen), and excessive use of distilled spirits, though beer and fermented liquors seemed to have an antiscorbutic effect. Then he wrote "I come in the next place, to an additional and extremely powerful cause, observed at sea to occasion this disease and which concurring with the former in progress of time seldom fails to breed it. And this is, the want of fresh vegetables and greens; either as may be supposed, to counteract the bad effects of their before mentioned situation, or rather, and more truly to correct the quality of such hard dry food as they are obliged to make use of. Experience indeed sufficiently shows that as greens or fresh vegetables, with ripe fruits, are the best remedies for it, so they prove the most effectual preservatives against it. And the difficulty of obtaining them at sea, together with a long continuance in the moist sea air are the true causes of its so general and fatal malignity upon that element."

XII. Future Problems

MARY ELIZABETH REID

Although there is no field in which further investigations on vitamin C could not be made profitably, problems of special urgency involve studies on (1) the pathology of scurvy, (2) relation of the vitamin to phagocytic activity and fragility, (3) changes in the circulatory system, (4) mineral metabolism, and (5) interrelations with other vitamins.

a. The Pathology of Scurvy

Much of the discrepancy in the reports on the pathology of vitamin C deficiency is caused by the fact that some investigators studied the acute condition whereas others dealt with the chronic type. There has been a tendency to criticize the chronic studies because of the absence of some of the acute symptoms and the presence of other symptoms not found in the acute disease. It seems clear that studies of both types are necessary.

[75] J. Lind, Treatise on Scurvy, 2nd ed. A. Millar, London, 1757.

Much of the older work on scurvy should be repeated, using diets presumably adequate in all factors other than ascorbic acid. Symptoms on which there is lack of agreement, at least as to degree of development, are swelling of joints, accumulation of water in the body cavity, and marked enlargement of the adrenals. In much of the published work there has been no control of food intake. There is definite need of study of the effect of food restriction, particularly on gastrointestinal hemorrhages.

b. Relation of the Vitamin to Phagocytic Activity and Fragility

Further studies should undoubtedly be made in this field under a variety of conditions in an attempt to evaluate quantitatively possible special needs of the vitamin for combating different types of infections. Special attention should be directed to observing if, under conditions of inadequate ascorbic acid supply, the polymorphonuclear cells tend to remain in the circulating blood stream and have a lessened tendency to migrate to the tissues.

c. Changes in the Circulatory System

It is obvious that further effort should be made to determine the localization and causes of weakness of the blood vessel walls. How and from where does the hemorrhagic blood escape from the vessels? Can the red cells pass through the dilated capillary walls as do the white cells normally? An observation by Nungester and Ames[35] suggests this possibility, although it is also possible that actual rupture of blood vessels occurs. They found that intraperitoneal exudates from scorbutic animals were invariably contaminated with large numbers of red blood cells whereas none were found in exudates having vitamin C content above 0.45 mg. per 100 ml. By studying different stages of embryos from animals on scorbutigenic diets, what conclusions can be reached as to the relation of the vitamin to cell boundaries and intercellular cement of the capillaries? Is the thin-walled condition characteristic of blood vessels in advanced scurvy, particularly of the chronic type, preceded by a somewhat thick-walled condition as a possible consequence of imperfect organization of cell materials? Are the leucoblasts of scorbutic animals longer lived than those of normal animals?

d. Mineral Metabolism

Studies are needed to determine the relation of ascorbic acid to (1) the proportion of ionized to total calcium in the blood, (2) the effect of the level of calcium in the diet on the ascorbic acid requirement and (3) possible changes in the proportion of phosphate to carbonate in teeth and bones after administering the vitamin to scorbutic animals.

e. Interrelations with Other Vitamins

The relation of vitamin A to ascorbic acid synthesis requires further investigation with due consideration of such factors as food intake and presence or absence in the diet of substances stimulatory to ascorbic acid synthesis. Much remains to be done as to specificity of action of ascorbic acid in its interrelations with other vitamins, particularly with reference to possible effects on enzyme action.

f. Other Problems

We need to know much more about the amino acid and fat relations of ascorbic acid under conditions of controlled food intake. Regardless of a vast amount of work already completed in the field of enzyme and hormone interrelations of the vitamin, not many, if any, of the problems have been settled; hence opportunities for future investigation appear to remain limitless.

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VITAMIN B_{12}

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I. Nomenclature and Formulas

ROBERT S. HARRIS

Accepted names:	Vitamin B_{12}
	Cyanocobalamin (B_{12})
	Hydroxocobalamin (B_{12a} and $_{12b}$)
Obsolete names:	Animal protein factor
	Erythrotin
	Factor X
	Physin
	Zoopherin
Empirical formula:	(?) $C_{63}H_{84}N_{14}O_{14}PCo$
Chemical name:	Not yet determined

Structural formula: see p. 411

II. Chemistry

DONALD E. WOLF and KARL FOLKERS

The interest in an anti-pernicious anemia factor dates back to the discovery[1] in 1926 that pernicious anemia could be controlled therapeutically with whole liver. Soon after this epoch-making discovery, various groups of investigators began to fractionate liver extracts for the purification of the anti-pernicious anemia principle.[2]

A. ISOLATION

The present review begins with a report[3] in 1948 on the isolation of vitamin B_{12} as a red crystalline substance. The crystallization of vitamin B_{12} was the consummation of a long program of investigation. The crystalline vitamin B_{12} possessed a bioactivity[3, 4] for *L. lactis* Dorner of 11×10^6 units per milligram, and 0.000013 γ per milliliter of culture medium was capable of supporting half-maximal growth under the conditions used. The new vitamin was found to be clinically active when a single dose of 3 to 6 γ was injected intramuscularly in patients with pernicious anemia.[5]

The isolation of vitamin B_{12} was described later by several groups of investigators. In each case, the isolation involved extensive use of various types of chromatography. Liver extract[6, 7] or the extract proteolyzed with papain was subjected to repeated adsorption on charcoal and elution with hot 65% alcohol. This type-step was followed by partition chromatography on damp silica columns. The liver extractives were applied to the silica columns in dry butanol, and the columns were developed with butanol containing about 11% water. Under these conditions, the partition chromatograms readily showed three colored zones: (1) a fast-moving yellow zone which could be washed from the column by continued development; (2) a pink zone which moved slowly down the column; and (3) a brown zone which remained at the top of the column. By clinical trials, it was found that nearly all the anti-pernicious anemia activity resided in the pink zone which could be eluted with 50% ethanol. Variations of this method involved

[1] G. R. Minot and W. P. Murphy, *J. Am. Med. Assoc.* **87**, 470 (1926).

[2] Y. SubbaRow, A. B. Hastings, and M. Elkin, *Vitamins and Hormones* **3**, 237 (1945).

[3] E. L. Rickes, N. G. Brink, F. R. Koniuszy, T. R. Wood, and K. Folkers, *Science* **107**, 396 (1948).

[4] M. S. Shorb, *Science* **107**, 397 (1948).

[5] R. West, *Science* **107**, 398 (1948).

[6] E. L. Smith, *Nature* **161**, 638 (1948); E. L. Smith, L. F. J. Parker, *Proc. Biochem. Soc. Biochem. J.* **43**, VIII (1948).

[7] D. Hodgkin, M. W. Porter, and R. C. Spiller, *Proc. Roy. Soc. (London)* **B136**, 609 (1949); K. H. Fantes, J. E. Page, L. F. J. Parker, and E. L. Smith, *Proc. Roy. Soc. (London)* **B136**, 592 (1949).

the use of other solvent mixtures such as *n*-or isopropanol containing 10 to 20 % water; the separation of pink zones was satisfactory but little superior to the separation with wet butanol. The pink-zone material was further fractionated by repetition of partition chromatography and by fractional precipitation with ammonium sulfate.

For other partition chromatography, starch could be used instead of silica. On starch columns, the pink fractions from both proteolyzed and non-proteolyzed liver extracts separated into two pink zones with R_f values of about 0.5 and 1.2, respectively. Non-proteolyzed liver gave mainly the slow-moving component; the faster moving fraction was apparently the product of autolysis of the liver extracts.

Treatment of the pink-zone fractions with trypsin, followed by chromatography on silica or charcoal, gave further purification, and then crystallization from aqueous acetone was sometimes possible. It was found advantageous to precipitate the red pigment with phosphotungstic acid, before final crystallization from aqueous acetone.[7]

It was observed[8] that purification of vitamin B_{12} from liver extracts was aided by *n*-butanol extraction of aqueous solutions containing fairly high concentrations of ammonium sulfate. Chromatography with bentonite or aluminum silicate columns was then used for final stages of purification. The sharply defined pink bands were eluted, and these fractions were found to crystallize from aqueous acetone.

Investigation of other sources of vitamin B_{12} led to the discovery that it is present in numerous natural materials, and particular interest is attached to fermentation broths.[9] The culture broths of strains of *Mycobacterium smegmatis*, *Lactobacillus arabinosus*, and *Bacillus subtilis*, and of several *Streptomyces* species such as *S. roseochromogenus*, *S. griseus*, and *S. antibioticus*, have all been found to show vitamin B_{12} activity. From one of these culture broths, produced by a strain of *S. griseus*, crystalline vitamin B_{12} was isolated.[9]

In the course of work on the isolation of vitamin B_{12} from natural sources, recourse has been made to paper strip chromatography. A novel method has been devised[10] in which concentrates containing vitamin B_{12} are subjected to partition chromatography on paper strips developed with water-saturated *n*-butanol. The developed strip is then dried and applied to the surface of a plate of nutrient agar seeded with *L. lactis* Dorner for about 10 minutes. Incubation of the plate overnight leads to a pattern of growth with "zones of exhibition," which indicate the presence of active constituents.

[8] B. Ellis, V. Petrow, and G. F. Snook, *J. Pharm. Pharmacol.* **1**, 60 (1949).

[9] E. L. Rickes, N. G. Brink, F. R. Koniuszy, T. R. Wood, and K. Folkers, *Science* **108**, 634 (1948).

[10] W. F. J. Cuthbertson and E. L. Smith, *Biochem. J.* **44**, V (1949); E. L. Smith, W. F. J. Cuthbertson, A. Walker, and K. A. Lees, *Federation Proc.* **9**, No. **1**, 230 (1950).

In this way, the presence of four active factors in liver extracts was demonstrated.[10] Two of these are evidently the colored fractions previously described[6] (vitamin B_{12} and a closely related factor); another is evidently thymidine. By somewhat similar procedures[11] involving paper chromatography and tube assay, the presence in crude extracts of five active constituents other than vitamin B_{12} has been recognized. These appeared to be desoxyribosides.[10-12]

As a result of further study of natural sources of vitamin B_{12}, other closely related substances with similar activity were discovered. When extractives from the fermentation broths of *Streptomyces aureofaciens* were subjected to chromatography on charcoal followed by elution and chromatography on silicic acid columns, vitamin B_{12} and a second red crystalline compound were isolated.[13] This second red compound, named vitamin B_{12b}, was closely related to vitamin B_{12} in physical and biological properties, but differed in certain characteristics.[14, 15] Crystalline vitamin B_{12b} has also been isolated from neomycin fermentations.[16] In the isolation procedure, the fermentation medium was acidified and heated to extract the active substance from the mycelium; the active substance was then adsorbed on charcoal. After elution the purity was increased by solvent partition through a series of separations. These were followed by a series of chromatographic adsorptions on charcoal; the purified eluates yielded crystalline vitamin B_{12b} from aqueous acetone.

From *S. griseus*, two other closely related substances have been isolated in crystalline form and called vitamins B_{12c} and B_{12d};[17] they appeared to be equally active clinically with vitamin B_{12} in the treatment of pernicious anemia. Vitamin B_{12d} was later found to be identical with vitamin B_{12b}.[18]

The literature has become somewhat confused by the nomenclature used in publications on these various modifications of vitamin B_{12}. The discussion of the chemical nature of these closely related compounds in the section on constitution should serve to clarify this unfortunate confusion.

[11] W. A. Winsten and E. Eigen, *J. Biol. Chem.* **181**, 109 (1949).

[12] V. Kocher, P. Karrer, and H. R. Muller, *Z. Vitaminforsch.* **21**, 403 (1950).

[13] J. V. Pierce, A. C. Page, Jr. E. L. R. Stokstad, and T. H. Jukes, *J. Am. Chem. Soc.* **71**, 2952 (1949).

[14] J. V. Pierce, A. C. Page, E. L. R. Stokstad, and T. H. Jukes, *J. Am. Chem. Soc.* **72**, 2615 (1950).

[15] H. Lichtman, J. Watson, V. Ginsberg, J. V. Pierce, E. L. R. Stokstad, and T. H. Jukes, *Proc. Soc. Exptl. Biol. Med.* **72**, 643 (1949).

[16] W. G. Jackson, G. B. Whitfield, W. H. DeVries, H. A. Nelson, and J. S. Evans, *J. Am. Chem. Soc.* **73**, 337 (1951).

[17] W. K. Anslow, S. Ball, W. B. Emery, K. H. Fantes, E. L. Smith, and A. D. Walker, *Chemistry & Industry* **1950**, 574; E. L. Smith, *Lancet* **258**, 353 (1950).

[18] E. L. Smith, *Nature* **169**, 60 (1952); E. L. Smith, K. H. Fantes, S. Ball, D. M. Ireland, J. G. Waller, W. B. Emery, W. K. Anslow, and A. D. Walker, *Proc. Biochem. Soc. Biochem. J.* **48**, L (1951); E. L. Smith, *Chemistry & Industry* **1951**, 939.

B. CHEMICAL AND PHYSICAL PROPERTIES

Vitamin B_{12} is a dark-red crystalline compound which darkens to black at about 212° and does not melt up to 320°.[3, 9] The refractive indices of the crystals are 1.619 (α), 1.649 (β), and 1.659 (γ).[9] It is optically active; the rotations reported by two laboratories are $[\alpha]_{6563}^{23} = -59 \pm 9°$,[19] and $[\alpha]_{6438}^{20} = -110° \pm 10\%$.[7] The absorption spectrum of vitamin B_{12} in aqueous solution shows the characteristic bands: 278 mμ ($E_{1\,cm.}^{1\%} = 115$); 361 mμ ($E_{1\,cm.}^{1\%} = 204$); and 550 mμ ($E_{1\,cm.}^{1\%} = 63$)[19] (Fig. 1). The absorption spectrum is not markedly affected by small changes in pH. Magnetic susceptibility measurements of vitamin B_{12} indicated that it is diamagnetic and that it is a trivalent cobalt complex with octahedral d^2sp^3 bonding.[20] The infrared absorption spectrum has been published.[7] In neutral solution, vitamin

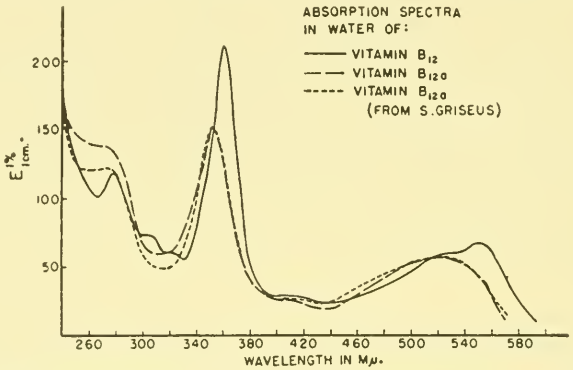


FIG. 1. Absorption spectra of vitamin B_{12} compounds. Reproduced with permission from *J. Am. Chem. Soc.* **73**, 355 (1951).

B_{12} shows a well-defined polarographic step with a half-wave potential at −1.53 v. (against the saturated calomel electrode) surmounted by a characteristic maximum.[7]

Early investigations on vitamin B_{12} revealed the presence of cobalt and phosphorus,[9, 21, 22] which were later shown to be present in the ratio of 1:1 in the vitamin B_{12} molecule.[19] Ebullioscopic determination of molecular weight indicated a value of 1490 ± 150.[19] This value is in good agreement with the minimum molecular weight of about 1300 which is the value determined by calculation based on a cobalt content of about 4.5%. Esti-

[19] N. G. Brink, D. E. Wolf, E. Kaczka, E. L. Rickes, F. R. Koniuszy, T. R. Wood and K. Folkers, *J. Am. Chem. Soc.* **71**, 1854 (1949).

[20] J. C. Wallmann, B. B. Cunningham, and M. Calvin, *Science* **113**, 55 (1951); H. Diehl, R. W. Vander Haar, and R. R. Scalock, *J. Am. Chem. Soc.* **72**, 5312 (1950); F. Grün and R. Menasse, *Experientia* **6**, 263 (1950).

[21] E. L. Smith, *Nature* **162**, 144 (1948).

[22] E. L. Rickes, N. G. Brink, F. R. Koniuszy, T. R. Wood, and K. Folkers, *Science* **108**, 134 (1948).

mation of the molecular weight on the basis of x-ray crystallographic data gave a range of 1360 to 1575.[7] Vitamin B_{12} appears to be a polyacidic base, as revealed by potentiometric titration in glacial acetic acid;[19] the basic groups are not detected by titration in aqueous solution. In the titration of vitamin B_{12} with perchloric acid in glacial acetic acid it was observed that excess reagent caused precipitation of an amorphous orange-colored precipitate.[23] Further investigation proved it to be a simple salt of the vitamin with 6 moles of perchloric acid. The vitamin B_{12} could be regenerated by treatment with water and the liberated perchloric acid titrated with alkali. The formation of this salt indicates the presence of six weakly basic groups in the vitamin.

Establishment of the molecular formula for vitamin B_{12} by conventional microanalyses is not readily feasible, because of the molecular size of the compound. The first suggested formulation of vitamin B_{12} was $C_{62}H_{86\text{-}90}N_{14}O_{13}PCo$ or $C_{63}H_{88\text{-}92}N_{14}O_{13}PCo$ for a sample dried in a weighing pig *in vacuo* at 100° for 2 hours.[19] Analyses on samples dried at room temperature gave results indicating a fairly high degree of hydration supporting a formula $C_{63}H_{97}N_{14}O_{20}PCo$.[7] More recently the perchloric acid salt of vitamin B_{12} has been analyzed and formulated as $C_{63}H_{84}N_{14}O_{14}PCo6HClO_4$.[23]

Reports on the stability of vitamin B_{12} under a variety of conditions have been published. As a generalization, it can be said that aqueous solutions of vitamin B_{12} are most stable in the pH range of 4 to 7 at normal temperatures.[24, 25] However, exposure to sunlight brings about loss of microbiological activity.[7] Autoclaving solutions at 115° for 30 minutes causes slight but significant decomposition.[24] Storage of aqueous solutions of vitamin B_{12} containing 0.5 % phenol or 0.3 % cresol brought about no detectable decomposition as determined by spectrophotometric or microbiological tests.[24] The presence of small concentrations of acid or base brings about significant loss of microbiological activity on storage.[7, 22] Vitamin B_{12} in 0.015 *N* sodium hydroxide solution (0.2 γ per milliliter) was microbiologically inactivated at room temperature as follows: 20 % (0.67 hour); 45 % (6 hours); 90 % (23 hours); 95 % (95 hours). It was inactivated in 0.01 *N* hydrochloric acid solution (10 γ per milliliter) as follows: 18 % (3 hours) 75 % (23 hours), 89 % (95 hours). The presence of oxidizing and reducing agents at the full range of pH values is accompanied by loss of microbiological activity.[7, 24, 25]

Aeration of a solution of vitamin B_{12} brought about an unknown chemical change which involved loss of the 280-mμ absorption maximum.[26] Chroma-

[23] J. F. Alicino, *J. Am. Chem. Soc.* **73**, 4051 (1951).
[24] F. Hartley, P. Stross, and R. E. Stuckey, *J. Pharm. Pharmacol.* **2**, 648 (1950).
[25] C. A. Lang and B. F. Chow, *Proc. Soc. Exptl. Biol. Med.* **75**, 39 (1950).
[26] H. G. Wijmenga, J. Lens, and A. Middelbeek, *Chem. Weekblad* **45**, 342 (1949) [*C. A.* **43**, 9193 (1949)].

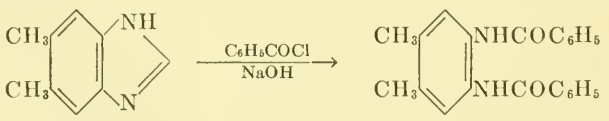
tography of the aerated solution allowed separation of two fractions which still possessed absorption maxima at 280 mμ.

C. CONSTITUTION

Soon after the initial announcements of the isolation of vitamin B_{12}, the investigation of the general nature or class of the compound began. Acid hydrolysis failed to indicate the presence of amino acids[19] but did reveal the presence of a single ninhydrin-reacting constituent.[8] The presence of phosphate ion and ammonia was recognized in the hydrolyzates.[27, 28] Analogy with other naturally occurring compounds possibly related metabolically to vitamin B_{12} suggested the possibility of a pterin, nucleotide, or polypyrrole structure. Evidence from physical and chemical experiments, particularly ultraviolet absorption data,[19] eliminated a pterin-like structure. Hydrolytic fragments characteristic of the purines have likewise been lacking.[8] However, alkaline fusion has produced pyrroles[19] as judged by their characteristic color reaction with *p*-dimethylaminobenzaldehyde (Ehrlich reagent). More extensive information on the exact nature of the pyrrole-like moiety will surely be forthcoming when appropriate methods to degrade the stable cobalt complex[8] have been devised. The biological relationship of vitamin B_{12} to the general hematopoetic metabolism seems to indicate that vitamin B_{12} is a polypyrrole related in some way to hemin or the bile pigments.

1. 5,6-Dimethylbenzimidazole

Despite the fact that acid hydrolysis of vitamin B_{12} fails to completely rupture the cobalt complex, it has been found to liberate certain portions of the molecule and, in doing so, to destroy its biological activity and bring about changes in the ultraviolet absorption spectrum. Hydrolysis of vitamin B_{12} in 6 *N* hydrochloric acid at 150° for 24 hours produced 5,6-dimethylbenzimidazole (I), which was isolated by continuous extraction of the alkaline hydrolyzate with chloroform.[29, 30] The general class identification of this degradation product was determined by appraisal of its molecu-



I II

lar composition and by comparison of its ultraviolet absorption spectrum with that of known benzimidazoles. The characteristic ultraviolet absorp-

[27] B. Ellis, V. Petrow, and G. F. Snook, *J. Pharm. Pharmacol.* **1**, 287 (1949).
[28] B. Ellis, V. Petrow, and G. F. Snook, *J. Pharm. Pharmacol.* **1**, 950 (1949).
[29] N. G. Brink and K. Folkers, *J. Am. Chem. Soc.* **71**, 2951 (1949).
[30] N. G. Brink and K. Folkers, *J. Am. Chem. Soc.* **72**, 4442 (1950).

tion spectrum of 5,6-dimethylbenzimidazole is shown in Fig. 2. A characteristic reaction of benzimidazoles with benzoyl chloride in alkaline solution gave the dibenzoyl derivative (II), which was finally identified by synthesis. The yield of 5,6-dimethylbenzimidazole amounted to about 70% of that

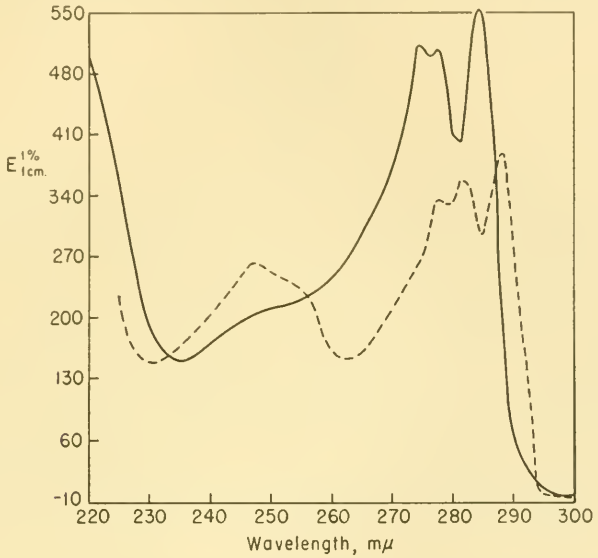
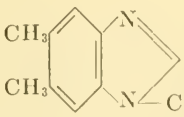


FIG. 2. Absorption spectrum of 5,6-dimethyl-benzimidazole in 95% ethanol. Solid line, in the presence of 0.01*N* hydrochloric acid; broken line, in the presence of 0.01*N* sodium hydroxide.

expected for one molar equivalent supporting the provisional structure (III).[30]



$C_{53-54}H_{77-81}N_{12}O_{13}PCo$

III

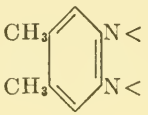
By an alternative method, the acid hydrolyzate of vitamin B_{12} was subjected to paper strip chromatography.[31] Observation of the paper chromatograms in ultraviolet light revealed the presence of three fluorescent spots, termed components α, β, and γ. Eluates of these fluorescent areas showed them to have similar ultraviolet absorption spectra. An extensive spectroscopic comparison with twenty-two different substituted benzimidazoles led to the conclusion that component γ was 5,6-dimethylbenzimidazole, and

[31] E. R. Holiday and V. Petrow, *J. Pharm. Pharmacol.* **1**, 734 (1949).

that components α and β (slower moving) were 1-substituted 5,6-dimethylbenzimidazoles.[31, 32]

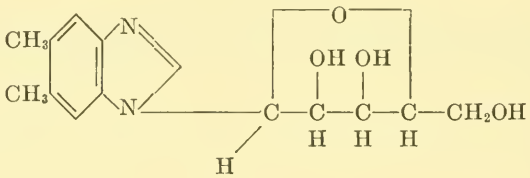
2. 1-α-D-Ribofuranosido-5,6-dimethylbenzimidazole

The presence of the 1,2-diamino-4,5-dimethylbenzene moiety (IV) in



IV

vitamin B_{12} and also in riboflavin could hardly escape attention. Furthermore, by analogy it was logical to suspect the presence of an N-pentose unit in combination with the 5,6-dimethylbenzimidazole. More careful investigation of the mild acid hydrolysis of vitamin B_{12} yielded 1-α-D-ribofuranosido-5,6-dimethylbenzimidazole (V) as a basic product with an absorption spectrum of the benzimidazole type. In addition, it gave a positive carbohydrate color test.[33] A crystalline picrate of the degradation product consumed 0.92 mole of periodate per mole, demonstrating a 1-pentofuranosido-5,6-dimethylbenzimidazole structure. The molecular formula of the product also agreed with this composition. The conditions which were used for the hydrolytic cleavage of the glycosidic linkage caused extensive de-

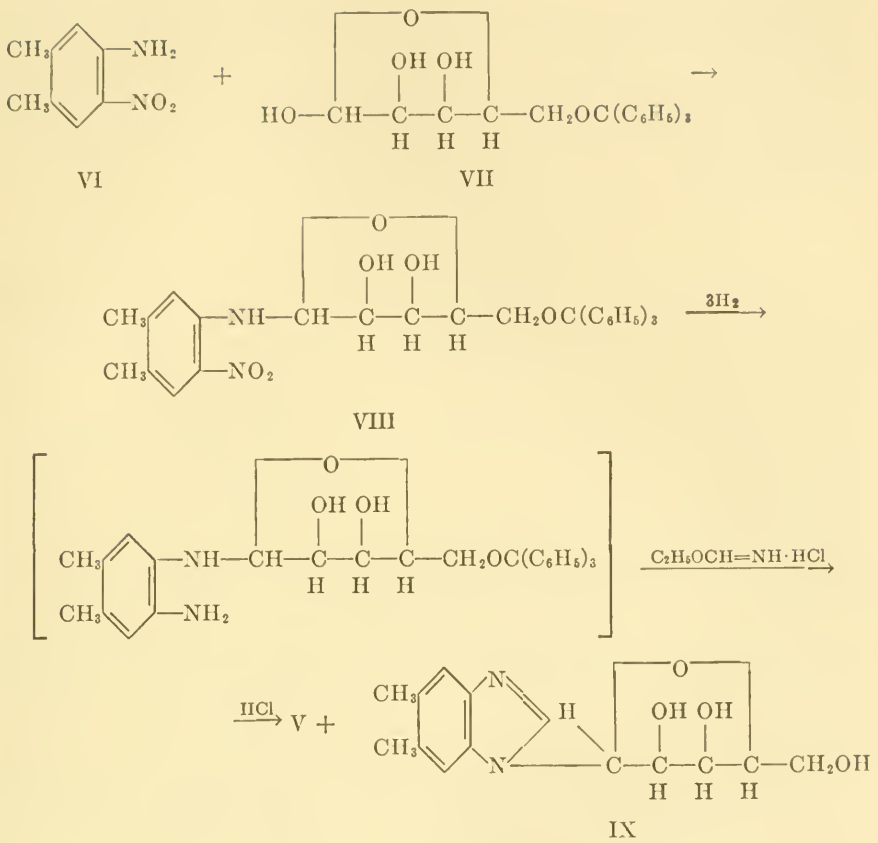


V

composition of the pentose. The structure of the product was proved by synthesis. 2-Nitro-4,5-dimethylaniline (VI) and 5-trityl-D-ribofuranose (VII) reacted to give 2-nitro-4,5-dimethyl-N-(5′-trityl-D-ribofuranosido)-aniline (VIII). Hydrogenation and condensation with ethyl formimino ether hydrochloride yielded 1-α-D-ribofuranosido-5,6-dimethylbenzimidazole (V), which was isolated as the picrate. Comparison of the picrates of the natural and synthetic products confirmed their identity. For convenience, compound V was given the designation α-ribazole.[33] The corresponding isomer, β-ribazole (IX), was also prepared.

[32] G. R. Beaven, E. R. Holiday, E. A. Johnson, B. Ellis, P. Mamalis, V. Petrow, and B. Sturgeon, *J. Pharm. Pharmacol.* **1**, 957 (1949).

[33] N. G. Brink, F. W. Holly, C. H. Shunk, E. W. Peel, J. J. Cahill, and K. Folkers, *J. Am. Chem. Soc.* **72**, 1866 (1950).



A detailed study has been made of the interrelationship of the three fluorescent substances, components α, β, and γ, which were previously mentioned.[31, 34] With the γ component synonymous with 5,6-dimethylbenzimidazole and the β component synonymous with 1-α-D-ribofuranosido-5,6-dimethylbenzimidazole, the nature of the α component remained in question. By analogy with other natural products, it was expected that α-ribazole is linked to phosphoric acid. It was found that mild hydrolysis of vitamin B_{12} (6 *N* hydrochloric acid at room temperature for 5 hours) brought about separation of not less than 0.7 mole of the α component.[34] Further hydrolysis of this component at elevated temperature gave a mixture of α component + β component + phosphate ion.[34, 35] The α compo-

[34] G. Cooley, B. Ellis, P. Mamalis, V. Petrow, and B. Sturgeon, *J. Pharm. Pharmacol.* **2**, 579 (1950).

[35] J. G. Buchanan, A. W. Johnson, J. A. Mills, and A. R. Todd, *Chemistry & Industry* **1950**, 426; J. G. Buchanan, A. W. Johnson, J. A. Mills, and A. R. Todd, *J. Chem. Soc.* **1950**, 2845.

nent appears therefore, to be phosphorylated ribazole, if no fragments remain undetected in the hydrolyzate. A ribazole phosphate was isolated as the barium salt. Since the ribazole phosphate fails to react with periodate, it is evident that it contains no α-glycol grouping, and the phosphoryl group then must be located at C_2 or C_3 of the ribose moiety.[35] The exact location of the phosphate grouping awaits further study.

3. D$_g$-1-Amino-2-propanol

At an early stage in the progress of the structural elucidation of the vitamin B_{12} molecule, it was noted that acid hydrolyzates, when subjected to paper strip chromatography, showed the presence of a single ninhydrin-reacting substance,[8] later found to be D$_g$-1-amino-2-propanol.[36]

An extended investigation of this substance by highly skilled paper strip chromatographic techniques followed.[37] Very small samples of vitamin B_{12} were subjected to hydrolysis in 2% hydrochloric acid at 100° for 6 hours. Chromatography on paper strips using a *n*-butanol-acetic acid mixture separated the ninhydrin-reacting fragment from the fluorescent benzimidazole derivatives. The ninhydrin-reacting substance was eluted and found to be transparent to ultraviolet light; it was concluded that the compound was an aliphatic base. By analogy with known naturally occurring substances, it was found that 2-aminopropanol showed identical behavior on paper strip chromatography.[28, 37] Further investigation, however, proved that the two substances were not identical.[38, 39] Oxidation of 2-aminopropanol with permanganate produced alanine which could be identified by the ninhydrin color reaction on a paper strip chromatogram. The ninhydrin-reacting substance from vitamin B_{12}, on the other hand, gave an oxidation product which was not alanine but which gave a yellow color with ninhydrin.

The identification of the ninhydrin-reacting substance as D$_g$-1-amino-2-propanol was accomplished by classical chemical means.[36] The acid hydrolyzate of vitamin B_{12} was extracted with butanol to remove the colored cobalt complex. The amino fragment was benzoylated and isolated as the dibenzoate. Since this compound was optically active, the structural possibilities were limited to compounds with an asymmetric center. The dibenzoate was hydrolyzed, and the solution containing the amino fragment was oxidized with sodium metaperiodate. From the oxidation mixture, acetaldehyde and formaldehyde were identified as dimedone derivatives. This information in conjunction with microanalytical data limited the possible structures to an aminopropanol with adjacent functional groups. Synthesis of D$_g$-1-amino-

[36] D. E. Wolf, W. H. Jones, J. Valiant, and K. Folkers, *J. Am. Chem. Soc.* **72**, 2820 (1950).
[37] B. Ellis, V. Petrow, and G. F. Snook, *J. Pharm. Pharmacol.* **1**, 735 (1949).
[38] G. Cooley, B. Ellis, V. Petrow, *J. Pharm. Pharmacol.* **2**, 128 (1950).
[39] G. Cooley, B. Ellis, V Petrow, *J. Pharm. Pharmacol.* **2**, 535 (1950).

2-propanol by lithium-aluminum hydride reduction of D$_g$-lactamide and isolation of the reduction product as the dibenzoate completed the proof of structure.

An extensive study has been made of the hydrolysis of vitamin B_{12} with relation to the order of liberation and stoichiometry of the various hydrolytic fragments. On the basis of quantitative colorimetric estimation on paper strip chromatograms, it has been reported that there are 2 moles of 1-amino-2-propanol to 1 mole of phosphorus in the vitamin B_{12} molecule.[40]

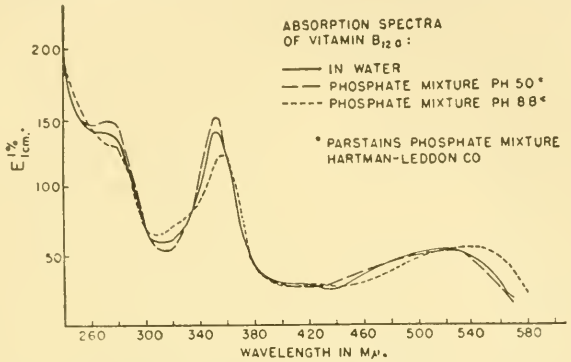


Fig. 3. Absorption spectra of vitamin B_{12}. Reproduced with permission from *J. Am. Chem. Soc.* **73**, 355 (1951).

4. Nature of the Cobalt Complex

Catalytic hydrogenation of vitamin B_{12} using platinum catalyst and hydrogen at 1 atm. caused the red color of the solution to change to dark brown. Exposure to air brought about restoration of the bright-red color characteristic of vitamin B_{12} solutions. A red crystalline product was isolated from the reaction mixture. Examination of its physical properties revealed that it was not vitamin B_{12} but a closely related substance which was named vitamin B_{12a}.[41, 42] Analyses revealed that the cobalt and phosphorus contents were about the same as in vitamin B_{12}. Several bands of the absorption spectrum had changed, and the spectrum was found to be more responsive to pH changes than is the spectrum of vitamin B_{12} (Figs. 1 and 3).[42] The spectrum of vitamin B_{12a} also showed a progressive change in the 315-mμ band with time, as shown in Fig. 4.[42] Vitamin B_{12a} was also isolated from culture broths of *Streptomyces griseus*[42] and is, therefore, a naturally occurring variant of the vitamin B_{12} group.

The biological activity of vitamin B_{12a} has been compared with that of vitamin B_{12} by various assay methods. Microbiological assays using *L. lactis*

[40] E. Chargaff, C. Levine, C. Green, and J. Kream, *Experientia* **6**, 229 (1950).

[41] E. A. Kaczka, D. E. Wolf, and K. Folkers, *J. Am. Chem. Soc.* **71**, 1514 (1949).

[42] E. A. Kaczka, R. G. Denkewalter, A Holland, and K. Folkers, *J. Am. Chem. Soc.* **73**, 335 (1951).

and *L. leichmannii* show approximately equivalent activity with that of vitamin B_{12}. In the "animal protein factor" assay with rats, the activity of vitamin B_{12a} is equivalent to that of vitamin B_{12}. Using chicks as the assay animals, the activity is approximately 50% of the activity of vitamin B_{12}. Clinical trials with vitamin B_{12a} in cases of Addison's pernicious anemia, tropical sprue, nutritional macrocytic anemia, and megaloblastic anemia of infancy have demonstrated the effectiveness of vitamin B_{12a} in producing a rise in reticulocytes, red blood cells, white blood cells, platelets, and hemoglobin and promoting a return of the bone marrow to normal.[42] As regards the quantitative dosage relationship between vitamins B_{12} and B_{12a} for

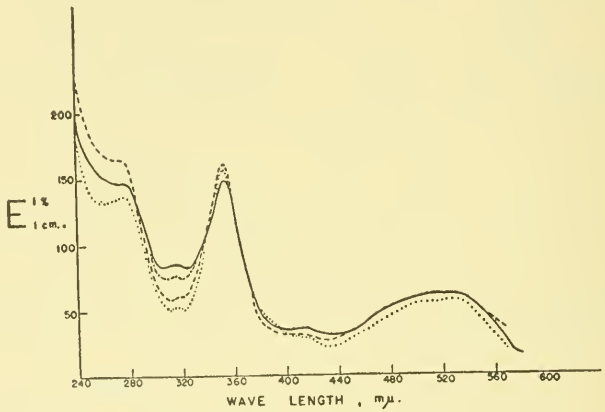


FIG. 4. Spectra in water of vitamin B_{12a} after drying *in vacuo* at 100° for 2 hours: ————, solution at t = *ca.* 1 minute; —·—·—, solution at t = ca. 15 minutes; ----------, solution at t = 24 hours; ..... spectrum of vitamin B_{12b} (Lederle NP-92-58-4) after drying at 100° for 2 hours *in vacuo*; water solution at t = over 30 minutes. Reproduced with permission from *J. Am. Chem. Soc.* **73**, 355 (1951).

human beings, exact data are lacking because of extreme variability from patient to patient; these vitamins appear to be essentially equivalent.

The isolation of crystalline vitamin B_{12b} from culture broths of *Streptomyces aureofaciens*[13] and also from neomycin fermentation broths[16] has been mentioned. A number of papers have appeared describing its isolation[13-15] and also its formation from vitamin B_{12} by catalytic hydrogenation.[43] Some early confusion appeared in the literature as to the relationship between the two modifications, vitamins B_{12a} and B_{12b}. It was evident later that the two substances are identical on the basis of all chemical and physical comparisons.[42, 44] Part of the misunderstanding appeared to come

[43] J. A. Brockman, J. V. Pierce, E. L. R. Stokstad, H. P. Broquist, and T. H. Jukes, *J. Am. Chem. Soc.* **72**, 1042 (1950).

[44] H. G. Wijmenga, W. L. C. Veer, and J. Lens, *Biochem. et Biophys. Acta* **6**, 229 1950).

from the fact that differentiation was based on preliminary tests of biological activities rather than the more exact classical chemical and physical comparisons. When samples were compared by these latter methods, it became evident that the designations vitamins B_{12a} and B_{12b} were two names for the same substance (see Fig. 4). Other forms of the vitamin B_{12} group from isolation programs, vitamins B_{12c} and B_{12d} (later retracted[18]), have been described,[17] and in the light of present knowledge many more forms may exist. Fortunately, the early complexity of the forms has been greatly simplified by a better understanding of the nature of the cobalt complex in this group of compounds.

Vitamin B_{12} is a cyanocobalt coordination complex in which the coordinatively bound cyano group is replaceable by other ions[45, 46] or molecules. The number of vitamin B_{12} modifications possible is then limited only by the number of ions or molecules which can replace cyanide in the cobalt coordination complex. Treatment of vitamin B_{12} with hydrochloric acid or aqueous oxalic acid liberates hydrogen cyanide whereas sulfuric acid fails to bring about its formation. This is evidently due to the greater coordination tendency of chloride and oxalate ions as compared to sulfate. In the formation of vitamin B_{12a} (B_{12b}) the cyanide ion is evidently liberated and replaced by a hydroxo group; in confirmation, it has been found that vitamin B_{12a} in aqueous solution has a pH of about 9 and behaves on titration as a weak base.[46] Further proof of the correctness of this interpretation is the fact that treatment of vitamin B_{12a} (B_{12b}) with cyanide ions brought about its conversion to vitamin B_{12}.[44, 46-48] On the basis of the evident nature of the cobalt complex in vitamin B_{12}, a system of nomenclature has been devised which can be applied to the modifications of vitamin B_{12}.[46] Under this system, the name *cobalamin* designates all the vitamin B_{12} molecule except the cyano group. Vitamin B_{12} is then cyanocobalamin, vitamin B_{12a} (B_{12b}) is hydroxocobalamin, and vitamin B_{12c} is nitritocobalamin.[18] Other modifications can be readily designated by this system. Chloro-, bromo-, sulfato-, cyanato- thiocyanato-, and nitrocobalamin have been described.[46, 49, 50] A sulfur-containing cobalamin of unknown group identity results from the reaction of cyanocobalamin with hydrogen sulfide.[46, 49]

Another naturally occurring modification of vitamin B_{12}, pseudovitamin

[45] N. G. Brink, F. A. Kuehl, and K. Folkers, *Science* **112**, 354 (1950).

[46] E. A. Kaczka, D. E. Wolf, F. A. Kuehl, and K. Folkers, *Science* **112**, 354 (1950).

[47] B. Ellis, V. Petrow, G. H. Beaven, E. R. Holiday, and E. A. Johnson, *J. Pharm. Pharmacol.* **2**, 735 (1950).

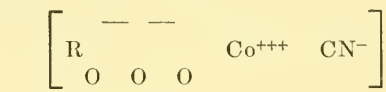
[48] W. L. C. Veer, J. H. Edelhausen, H. G. Wijmenga, and J. Lens, *Biochim. et Biophys. Acta* **6**, 225 (1950).

[49] E. A. Kaczka, D. E. Wolf, F. A. Kuehl, and K. Folkers, *J. Am. Chem. Soc.* **73**, 3569 (1951).

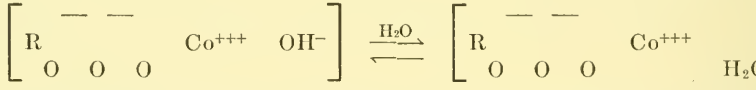
[50] R. P. Buhs, E. G. Newstead, and N. R. Trenner, *Science* **113**, 625 (1951).

B_{12}, has been isolated.[51] An unidentified organism isolated from bovine rumen contents when grown under anaerobic conditions has been found to produce a mixture of cobalt-containing pigments belonging to the vitamin B_{12} group.[51] From this mixture, two compounds have been isolated in crystalline form. Their microbiological growth activity, measured by *L. leichmannii* and *L. lactis* Dorner, is about the same as that of vitamin B_{12}. In the chick and the rat, however, they are inactive. One of these, pseudovitamin B_{12}, appears to differ from vitamin B_{12} in containing adenine instead of 5,6-dimethylbenzimidazole in the nucleotide part of the molecule.[51] Acid hydrolysis of pseudovitamin B_{12} produced D$_g$-1-amino-2-propanol, phosphate, ammonia, adenine, and hypoxanthine. The hypoxanthine apparently results from hydrolytic deamination of adenine.

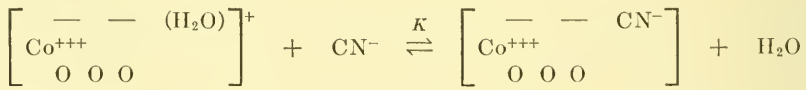
Various ideas as to the more exact structure of the cobalt coordination complex in vitamin B_{12} have been expressed as a result of structure work thus far completed. A generalized expression for the vitamin B_{12} molecule as a cyanocobalt coordination complex has been represented as follows:[49]



The cyano group contributes one negative charge and satisfies one coordination position; the two minus signs and the ciphers represent another group or groups which furnish two negative charges and three electron pairs, respectively, to satisfy the remaining five coordination bonds. In the case of hydroxocobalamin, the following equilibrium has been suggested to exist:



The vitamin B_{12}–B_{12a} equilibrium has been represented by the equation below:



Polarographic examination of this equilibrium failed to demonstrate detectable amounts of cyanide ion, which indicates that the equilibrium constant K must be very large.[50]

In more general form the equilibrium becomes:

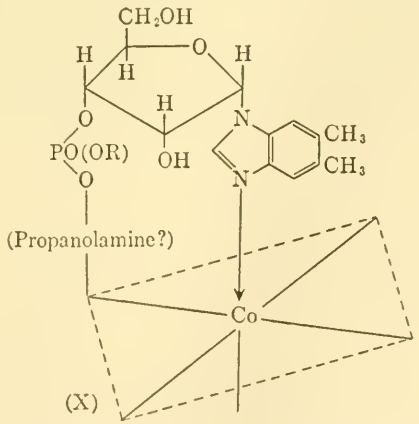
[51] J. J. Pfiffner, D. G. Calkins, R. C. Peterson, O. D. Bird, V. McGlohon, and R. W. Stipek, *Abstr. 120th Meeting Am. Chem. Soc.* p. 22C (1951); H. W. Dion, D. G. Calkins, and J. J. Pfiffner, *J. Am. Chem. Soc.* **74**, 1108 (1952).

$$\left[\begin{matrix} & - & - & (H_2O) \\ Co^{+++} & & & \\ & O & O & O \end{matrix}\right]^{+} + X^{-} \rightleftharpoons \left[\begin{matrix} & - & - & X^{-} \\ Co^{+++} & & & \\ & O & O & O \end{matrix}\right] + H_2O$$

Other modifications of vitamin B_{12} are readily prepared by treatment of hydroxocobalamin with the desired reagent as, for example, hydrochloric acid to form chlorocobalamin, and hydrobromic acid to give bromocobalamin.[49]

The photochemical reaction which liberates CN^- from the cyanocobalamin complex has been described as one reaction which leads to the formation of hydroxocobalamin,[48] and it is claimed that irradiation in the 365-mμ range is specific for this photochemical conversion. An excellent method has been developed for the quantitative determination of cyanocobalamin based on the measurement of CN^- liberated by the use of visible light.[52] It was found that irradiation of solutions of cyanocobalamin with visible light from an electric bulb catalyzed the liberation of hydrogen cyanide which could be swept out by a stream of nitrogen and determined colorimetrically. The reaction is reversible, and addition of cyanide ions forms first an unstable purple complex involving two additional cyanide ions; when the solution is acidified or the excess cyanide ion is otherwise removed, cyanocobalamin results.[48, 52, 53]

An extended study of the ultraviolet absorption spectrum of vitamin B_{12} and also of the spectral contribution of the benzimidazole moiety has led to certain suggestions as to the spatial arrangement of the benzimidazole nucleus in the cobalt complex.[54] These spectroscopic data, which bear only upon the coordination features, have led to the proposed formulation (X);



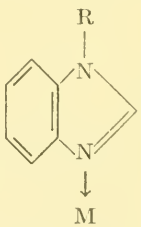
[52] G. E. Boxer and J. C. Rickards, *Arch. Biochem.* **30**, 372, 382, 392 (1951).

[53] G. H. Beaven, E. R. Holiday, E. A. Johnson, B. Ellis, and V. Petrow, *J. Pharm. Pharmacol.* **2**, 733, 944 (1950).

the data do not contribute to the knowledge of the linkage of phosphate to the ribose moiety. The descriptive formula X suggests a twofold attachment of the benzimidazole glycoside residue to the cobalt-containing macrofragment of the vitamin B_{12} molecule, which is visualized as a planar structure similar in spacial arrangement to the porphyrins. The formulation implies the existence of a stable coordinate link between the benzimidazole glycoside residue and the planar cobalt complex of a character not previously recorded.[54] It was inspired by the observation that the two bands in the absorption spectrum of vitamin B_{12} at λ = 2885 A. and 2785 A. (Fig. 1) are characteristic of 5,6-dimethylbenzimidazole and also its ribofuranoside and ribofuranoside phosphate which were identified as acid hydrolysis products of vitamin B_{12} (Fig. 2). An extensive research program has been carried out on the chemistry of the benzimidazoles in support of this formulation.

It was observed that a number of synthetic 5,6-dimethylbenzimidazole glycosides possess absorption spectra indistinguishable from each other and from those of paper strip components α and β (α-ribazole phosphate and α-ribazole, respectively). All these compounds show a well-defined "notch" in their absorption spectra at λ = 2850 A. in dilute acid and λ = 2880 A. in dilute alkali.[53] The absorption spectrum of vitamin B_{12} shows only an inflection in this region which is unchanged in position over the pH range 2 to 12.[53] If the absorption band of vitamin B_{12} in this region is due to the presence of the 5,6-dimethylbenzimidazole grouping, then some structural alteration must be postulated for this moiety which would account for its change of absorption behavior.

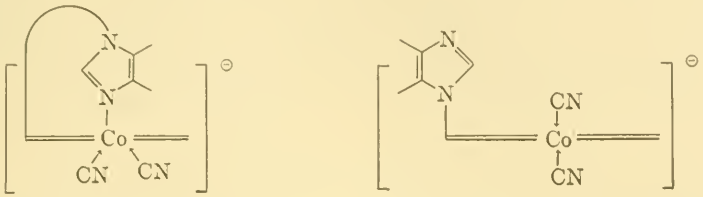
Two principal lines of evidence have been suggested to account for this anomaly. The first of these is the observation that the absorption spectrum of 5,6-dimethylbenzimidazole becomes much more like that of vitamin B_{12} when the benzimidazole enters into a coordination complex with a metal ion.[53] The second line of evidence is based on the observation that, when



(M = Metal)

[54] G. Cooley, B. Ellis, V. Petrow, G. H. Beaven, E. R. Holiday, and E. A. Johnson, *J. Pharm. Pharmacol.* **3**, 271 (1951); M. T. Davies, P. Mamalis, V. Petrow, and B. Sturgeon, *J. Pharm Phamacol.* **3**, 420 (1951)

vitamin B_{12} forms a "vitamin B_{12}-cyanide complex" with excess cyanide ion, its absorption spectrum breaks into a doublet with a "notch" at $\lambda = 2885$ A., characteristic of the benzimidazole absorption spectrum.[53] This is interpreted in one of two representations as indicated below and explained by assuming that the combination of the excess cyanide ion with the cobalt



leads to an increased electronegativity of the cobalt atom and a corresponding decrease in the electronic contribution of the N^3-benzimidazole nitrogen to the cobalt electronic cloud.[53] This effect is then said to allow for the more "normal" contribution of the 5,6-dimethylbenzimidazole grouping to the absorption spectrum.

The position of the D$_g$-1-amino-2-propanol in the representation of the cyanocobalamin molecule (X) has not been determined. However, some light has been shed on the problem by the determination of the order of hydrolytic liberation of the various degradation products.[34] Acid hydrolysis of cyanocobalamin at room temperature for 5 hours liberated 0.7 mole of ribazole phosphate. Further contact with 6 *N* hydrochloric acid for not less than 18 hours was necessary to liberate the D$_g$-1-amino-2-propanol.[34] Therefore, it seemed that, since the two moieties are not liberated simultaneously, the phosphate cannot be esterified with the propanolamine. Some other location must be found for this 3-carbon portion.

The graphic representation of cyanocobalamin[53] described above is not without some unsettled features. It has been found that cyanocobalamin reversibly binds more than one cyanide ion.[55] However, there is still strong evidence for the hypothesis that the benzimidazole chromophore is coordinated to the cobalt atom.[55a] The differential absorption spectra of vitamin B_{12} versus vitamin B_{12} "red fragment" (resulting from hydrolysis which is known to liberate the α-ribazole phosphate) have been plotted at pH 4, at pH 10, and at pH 10 plus potassium cyanide. The resulting curves then are the absorption spectra of the portion of the vitamin B_{12} molecule absent in the red fragment. The differential curve measured in the presence of potassium cyanide is very similar to that of the α-ribazole phosphate and strongly indicates the presence of the latter in the postulated position.

[55] J. B. Conn, S. L. Norman, and T. G. Wartman, *Science* **113**, 658 (1951).
[55a] G. H. Beaven and E. R. Holiday, *J. Pharm. Pharmacol.* **4**, 342 (1952).

5. Miscellaneous Degradation Reactions

The oxidation of vitamin B_{12} with hydrogen peroxide under mild alkaline conditions[56] is reported to give a neutral and an acidic product. Both are red crystalline compounds with absorption spectra identical to that of vitamin B_{12}, but with only a fraction of its microbiological activity. Dilute alkali treatment of vitamin B_{12} in the absence of light gave an acidic red crystalline product; and potassium persulfate gave a neutral red crystalline product. Both had identical ultraviolet absorption spectra similar to that of vitamin B_{12}, but they had very low microbiological activity.

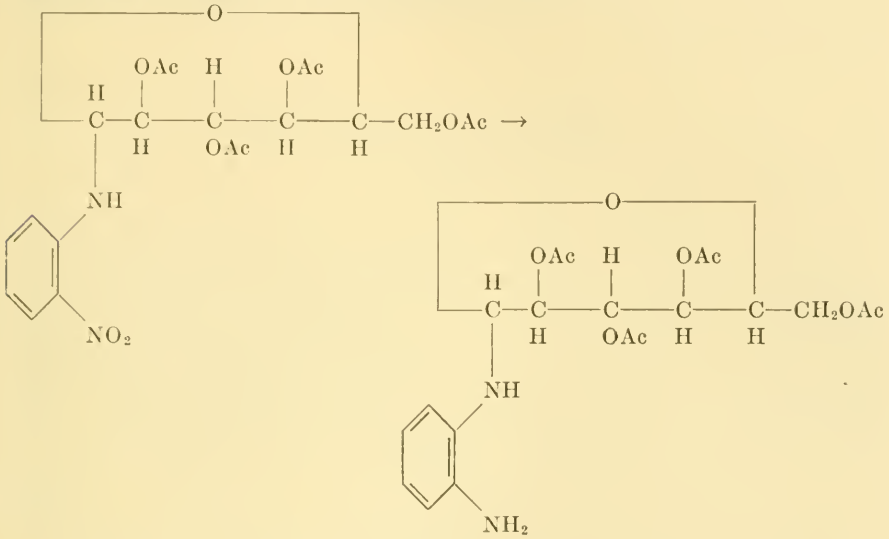
D. SYNTHESIS

The structure of vitamin B_{12} is not yet fully elucidated, and no synthesis of this vitamin has been accomplished. Synthesis of the various degradation products of vitamin B_{12} has been accomplished. In one particular instance, this has led to more extensive work. The synthesis of 5,6-dimethylbenzimidazole was carried out at the time this compound was isolated.[29, 30] Tests of its biological activity followed shortly.[57] Both 5,6-dimethylbenzimidazole and the corresponding diamine, 1,2-dimethyl-4,5-diaminobenzene, were inactive for *Lactobacillus lactis* Dorner in the assay for vitamin B_{12} activity, but both compounds possessed vitamin B_{12}-like activity for rats on a diet devoid of animal protein and containing 0.25% of thyroid powder.[57] Daily quantities of 2 to 5 mg. of 5,6-dimethylbenzimidazole and 2 to 3 mg. of 1,2-diamino-4,5-dimethylbenzene were comparable to 0.125 to 0.250 γ of vitamin B_{12}. A series of other benzimidazole-type compounds were synthesized and tested for vitamin B_{12}-like activity. Only 5-methylbenzimidazole possessed significant growth activity; 2,5-dimethylbenzimidazole appeared to act as a growth depressant. The following gave no significant growth response on the test diet in rats: benzimidazole, 1-methyl-, 2-methyl-, 4-methyl-, and 4,6-dimethylbenzimidazole.

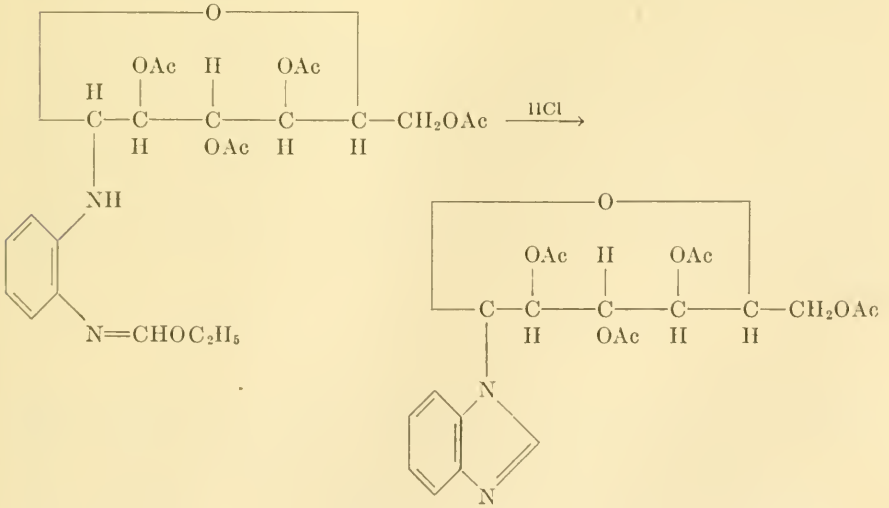
The synthesis of 1-α-D-ribofuranosido-5,6-dimethylbenzimidazole (α-ribazole),[33] the hydrolysis product isolated from vitamin B_{12}, has been reviewed in the section of this chapter dealing with constitution. A program of synthetic work on the glycosides of benzimidazole has been carried out because of the interest in the possible biological activities of these compounds.[58, 59, 60] The syntheses involved the glycosidation of an *o*-nitroaniline by boiling with the hexose or pentose sugars in ethanol in the

[56] O. Schindler, *Helv. Chim. Acta* **34**, 101 (1951).
[57] G. Emerson, N. G. Brink, F. W. Holly, F. Koniuszy, D. Heyl, and K. Folkers, *J. Am. Chem. Soc.* **72**, 3084 (1950).
[58] P. Mamalis, V. Petrow, and B. Sturgeon, *J. Pharm. Pharmacol.* **2**, 491 (1950).
[59] P. Mamalis, V. Petrow, and B. Sturgeon, *J. Pharm. Pharmacol.* **2**, 503 (1950).
[60] P. Mamalis, V. Petrow, and B. Sturgeon, *J. Pharm. Pharmacol.* **2**, 512 (1950).

presence of ammonium chloride. Glycosides were prepared from D-glucose, D-mannose, D-galactose, L-arabinose, D-xylose, D-ribose, and L-rhamnose as sugars; the aglycones were *o*-nitroaniline, *m*-nitro-*p*-toluidine, 3- and 5-nitro-*o*-4-xylidines.[58] The glycosides formed in this reaction were usually a mixture of two isomers; these were acetylated and subjected to catalytic reduction as shown below for the glucoside.



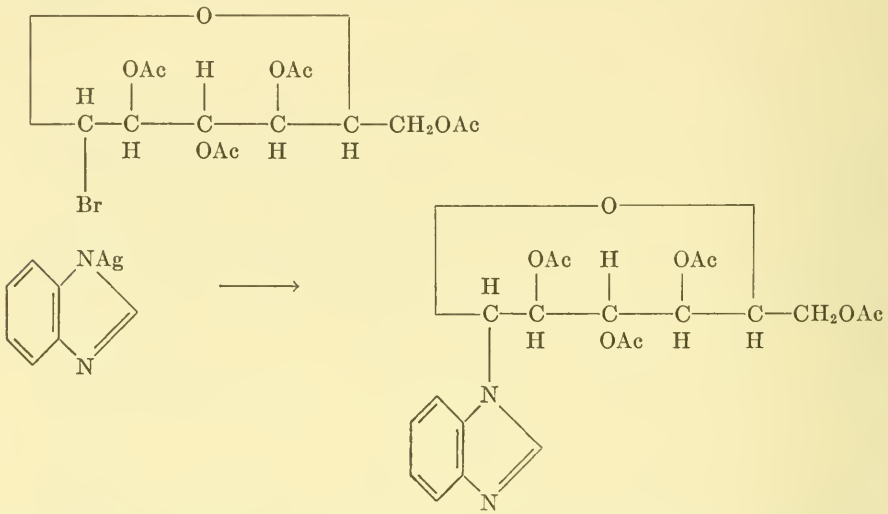
Reduction of certain isomeric mixtures brought about the formation of a single compound. Treatment with ethyl orthoformate gave the cor-



responding 2-ethoxymethylene-*o*-phenylenediamine tetraacetyl-D-glucoside. Conversion to the benzimidazole was accomplished by hot dilute hydrochloric acid.[59]

Hydrolysis of the acetyl groups was accomplished by heating the tetraacetate in 6 *N* hydrochloric acid at 100° for 3 hours.

An alternative method for the preparation of a benzimidazole glycoside involved the reaction of the silver salt of the benzimidazole with the acetobromosugar as follows:



This latter method was used for the confirmation of the structure of the benzimidazole glucosides.

The β-D-glycopyranosides of benzimidazole, 5-methyl-, 4,5-dimethyl-, and 5,6-dimethylbenzimidazole were prepared by these methods.[59]

A series of benzimidazole pentosides was prepared by the orthoformate condensation.[60] The following compounds were prepared: benzimidazole-1β-D-xylopyranoside; 5-methylbenzimidazole-1β-D-xylopyranoside; benzimidazole-1α-L-arabopyranoside; benzimidazole-1α-D-arabopyranoside; 5(or 6)-methylbenzimidazole-1α-L-arabopyranoside; 5,6-dimethylbenzimidazole-1α-L-arabopyranoside; 5,6-dimethylbenzimidazole-1-L-rhamnopyranoside.

1. Radioactive Modifications

Isotope-labeled vitamin B_{12} has been prepared to facilitate the determination or assay of vitamin B_{12}, and also to study its metabolism. Cobalt 60-labeled vitamin B_{12} has been prepared[61] by adding the cobalt 60 as the

[61] C. Rosenblum and D. T. Woodbury, *Science* **113**, 215 (1951); L. Chaiet, C. Rosenblum, and D. T. Woodbury, *ibid.* **111**, 601 (1950).

nitrate to the nutrient medium in which vitamin B_{12} is produced by fermentation. A product of a specific activity of $\simeq$ 67 μc. per milligram was obtained. It has also been possible to label the cyanide group [62] of cyanocobalamin by treating vitamin B_{12a} with $HC^{14}N$.

Direct neutron irradiation of vitamin B_{12} resulted in formation of the radioactive form containing cobalt 60.[63] The crystals were not visibly altered by irradiation, and the microbiological activity was retained.

III. Industrial Preparation

H. M. WUEST

The industrial history of vitamin B_{12} is nearly as dramatic as its isolation and early clinical use. As the compound was found in an industrial laboratory and proved to be of clinical value from the very beginning, it was only natural that it was practically available to the medical profession a short time after its isolation; four months after the first publication of the group around Karl Folkers (Rickes *et al.*, April, 1948[1],) Merck & Co., Inc., announced at the meeting of the Hematological Society in Buffalo that crystalline vitamin B_{12} was available for therapeutic purposes (August, 1948).

Today vitamin B_{12} plays an important role not only in the pharmaceutical industry but also in agriculture. The following forms are important:

a. Low concentrates, up to 15 mg. of B_{12} per pound = 0.0033 %, without or with antibiotics.

b. Medium and high concentrates, up to 1000 γ per gram = 0.1 % B_{12}.

c. Crystals, U.S.P. 95 % pure.

Form *a* is used only for the preparation of animal feed, *b* serves for oral therapeutic use, mainly for pan-vitamin mixtures, whereas *c* is the starting material for ampuled solutions (with 15 or 30 γ per milliliter).

Compared with the enormous scientific and clinical literature on B_{12}, the technical literature of its preparation is rather modest. However, considerable information can be collected from the patent descriptions.

Three sources of raw material may be considered as a starting point for the isolation of B_{12} as such or in the form of concentrates:

1. The mother liquors of the microbial formation of antibiotics like

[62] G. E. Boxer, J. C. Rickards, C. Rosenblum, and D. T. Woodbury, *Arch. Biochem.* **30**, 470 (1951).

[63] R. C. Anderson and Y. Delabarre, *J. Am. Chem. Soc.* **73**, 4051 (1951).

[1] E. L. Rickes, N. G. Brink, F. R. Koniuszy, T. R. Wood, and K. Folkers, *Science* **107**, 396 (1948).

streptomycin, aureomycin, and terramycin, after the removal of the antibiotic.

2. Cultures of microorganisms which produce B_{12} as the only valuable product, e.g., *B. megatherium.*
3. Activated sludge from sewage disposal.

At the time of this writing (September, 1953) source 1 provides almost the whole output of B_{12}; small amounts of concentrates are produced from source 2, whereas the use of activated sludge is still in a pilot plant stage.

Concentration of the Starting Material: 1 to 2 γ of B_{12} per milliliter of broth (or gram per cubic meter) is considered worth while for industrial extraction. The highest activity for B_{12} production reported in the literature is 8 γ per milliliter;[2] however, this figure was obtained by microbiological assay, and no microbiological method is entirely specific for B_{12}.

A. ISOLATION

1. From the Mother Liquors of Antibiotics

It is self-evident that a raw material which is a by-product and which yields a very valuable end product is always welcome. The producers of antibiotics with vitamin B_{12} as a by-product are in a favorable position.

The isolation of an active compound from a broth with a dilution as high as 1:1,000,000 is quite a technical achievement. It normally starts from the filtered broth after removal of the antibiotic and the vitamin B_{12} from the liquid phase by adsorption with charcoal,[3,4] calcium montmorillonite (fuller's earth), sodium montmorillonite (bentonite,)[5] or carboxylic ion exchange resins (Amberlite),[6] followed by elution with such diversified solvents as aqueous pyridine or α-picoline,[3,4] aqueous solutions of sodium cyanide or thiocyanate,[5] mixtures of acetone, water, and dilute hydrochloric acid,[6] and others. For further purification, countercurrent distribution between two liquid phases (e.g., *o*-cresol,[7] amylphenol,[8] or benzyl alcohol-water),[9] extraction from aqueous solution with a solid solvent

[2] D. Perlman, W. E. Brown, and S. B. Lee, *Ind. Eng. Chem.* **44**, 2000 (1952).

[3] F. A. Kuehl and L. Chaiet, (to Merck & Co., Inc.), U. S. Pat. 2,505,053 (April 25, 1950).

[4] E. L. Rickes and T. R. Wood, (to Merck & Co., Inc.), U. S. Pat. 2,563,794 (Aug. 7, 1951).

[5] S. Kutosh, G. B. Hughey, and R. Malcolmson, (to Merck & Co., Inc.), U. S. Pat. 2,626,888 (Jan. 27, 1953).

[6] W. Shive, (to Research Corp.), U. S. Pat. 2,628,186 (Feb. 10, 1953).

[7] N. G. Brink and T. R. Wood, (to Merck & Co., Inc.), U. S. Pat. 2,609,325 (Sept. 2, 1952).

[8] F. R. Koniuszy, N. G. Brink, and K. Folkers, (to Merck & Co., Inc.), U. S. Pat. 2,594,314 (April 29, 1952).

[9] N. G. Brink and F. J. Wolf, (to Merck & Co., Inc.), U. S. Pat. 2,607,717 (Aug. 19, 1952).

(2,4,5-trichlorophenol),[10] and precipitation as copper or zinc cyanide-vitamin B_{12} complex[11] may be used. Near the end chromatography on activated alumina[4, 12] and fractionated precipitation of the active substance in methanolic or ethanolic solution with acetone[4, 12] is recommended. The final crystallization takes place when an aqueous solution of the purified substance (50 % purity) is treated with acetone (10 mg. + 1 ml. of water + 10 ml. of acetone); upon standing, vitamin B_{12} separates from the solution in clusters of small red needles.[4]

Each step in the complicated series of operations increases the potency but includes a certain loss of active substance. A yield as high as 65 %, however, is claimed from the broth (1:1,000,000) to a concentrate of 5 % (U. S. Pat. 2,582,589, example 2); from there on the losses are much higher: U. S. Pat. 2,563,794 claims in example 6 only a yield of about 4 % in the form of crystals (14.1 mg. from 364 mg.), starting from a concentrate with 0.4 γ per gram.

The preparation of animal feed containing aureomycin and B_{12} is described by Jukes[13] (Aurofac, Lederle, containing 1.8 to 5.0 g. of aureomycin and up to 1.8 mg. of B_{12} per pound). A preparation of Pfizer, called TM 3 + 3, contains 3 g. of terramycin and 3 mg. of B_{12} per pound.

2. Fermentation Processes Producing Only B_{12}

Fermentation processes producing B_{12} without any other highly valuable product are economically justified only when they have definite advantages over the fermentation producing antibiotics at the same time. *Bacillus megatherium* as used by Lewis and coworkers[14, 15] shows the unusual features that propagation on a medium consisting of molasses, ammonia, and inorganic salts is already completed within 6 hours (compared with the usual three days), and that the vitamin-containing product is quantitatively recovered by centrifuging and drum drying, converting about 50 % of the weight of sugar used to bacterial solids. The pale tan, palatable, nontoxic, free-flowing, non-hygroscopic powder contains high nutritional value in addition to 15 mg. of B_{12} per kilogram.

The process is industrially used by one producer in the West (Pacific Yeast Co., Wasco, California). [16]

[10] R. G. Denkewalter, G. B. Hughey, and S. Kutosh, (to Merck & Co., Inc.), U. S. Pat. 2,635,985 (April 21, 1953).

[11] A. J. Holland, (to Merck & Co., Inc.), U. S. Pat. 2,621,144 (Dec. 9, 1952).

[12] H. H. Fricke, (to Abbott Laboratories), U. S. Pat. 2,582,589 (Jan. 15, 1952).

[13] T. H. Jukes, (to American Cyanamid Co.), U. S. Pat. 2,619,420 (Nov. 25, 1952).

[14] J. A. Garibaldi, K. Ijichi, J. C. Lewis, and J. McGinnis, (to the United States), U. S. Pat. 2,576,932 (Dec. 4, 1951).

[15] J. A. Garibaldi, K. Ijichi, N. S. Snell and J. C. Lewis, *Ind. Eng. Chem.* **45**, 838 (1953).

[16] *Chem. Eng.*, **59**, 244 (November, 1952).

3. Addition of Cobalt to the Fermentation

Vitamin B_{12} contains about 4 % cobalt. The natural cobalt content of the normal ingredients of the broth is very small. The addition of small amounts of cobalt salts to the broth increases the B_{12} production; the increase goes up to seventeen times the amount without addition of cobalt. The optimal concentration is about 2 parts of cobalt per million of the nutrient medium; 4 p.p.m. already decreases the simultaneous production of streptomycin to nearly one-fourth.[14, 17, 18]

4. B_{12} from Activated Sludge

Porges[19] and his group were the first to show that activated sludge from sewage disposal plants contains considerable amounts of B_{12}. No industrial firm, however, has tried to use this source. In June, 1953, the Sewerage Commission of Milwaukee, Wisconsin, announced[20] the erection of a pilot plant to this end, and shortly afterward a United States patent on the process was granted to Miner and Wolnak.[21] It seems likely that the process will have industrial success only when the enormous amounts of solid raw material are disposed of as a paying product (used as fertilizer, Milorganite).

B. PATENT SITUATION

About twenty United States patents protect methods of production and isolation of B_{12}, whereas the compound itself is protected in this country by a product claim (Rickes and Wood[4]), defining B_{12} clearly by its absorption spectrum and biological activity. Merck & Co., Inc., leads the field by owning the product claim and the addition of cobalt to the fermentation nutrient.[17]

C. PRODUCTION AND PRICES

In the short time of five years vitamin B_{12} has become one of the most important vitamins, therapeutically as well as economically. In 1951 in this country alone 48 lb. with a sales value of 11 million dollars were sold; the production in 1952 was 94 lb., and the sales totaled 61 lb. with an estimated value of 13 to 14 million dollars.

[17] T. R. Wood and D. Hendlin, (to Merck & Co., Inc.),U. S. Pat. 2,595,499 (May 6, 1952).

[18] The Distillers Company Ltd., Edinburgh, Swiss Pat. 287,713 (April 16, 1953).

[19] S. R. Hoover, L. B. Jasewicz, and N. Porges, *Science* **114**, 213 (1951).

[20] *Chem. Eng. News* **31**, 2689 (1953); *Drug Trade News*, manufact. section, p. 69 (July 6, 1953).

[21] C. S. Miner and B. Wolnak, (to Sewerage Commission of Milwaukee), U. S. Pat. 2,646,386 (July 21, 1953).

With the rapid technological development and the increased consumption, the original price of $12,500 per gram came down to about $300 per gram in the form of crystals and $50 per gram in the form of low concentrates (1953).

Even with this low price, vitamin B_{12} remains the highest priced organic compound on the open market. Therapeutically, however, vitamin B_{12} cannot be considered to be an expensive vitamin; the high clinical dose of 30 γ costs less than one cent in the form of the crystals.

D. PURITY OF THE COMMERCIAL PRODUCT

The requirements governing the purity of commercial vitamin B_{12} are described in U.S.P. XIV, p. 660 (official from November 1, 1950); for the official activity assay see third supplement p. 15 (January 1952).

IV. Biochemical Systems

THOMAS H. JUKES and WILLIAM L. WILLIAMS

A. VITAMIN B_{12} AND NITROGEN METABOLISM IN ANIMALS

A deficiency now known to be that of vitamin B_{12} was shown to be intensified in rats and chicks by raising the protein level of the diet.[1, 2] These early experiments were an indication of an involvement of vitamin B_{12} in the metabolism of compounds of nitrogen. Further relationships were reported in 1946 by Zucker and Zucker,[3] who found that rats on all-vegetable diets had high blood-urea levels and enlarged kidneys, and that stoppages in the urinary tract leading to hydronephrosis sometimes occurred. The feeding of liver abolished the symptoms and led to normal growth. Cary and Hartman[4] also found that rats which had been for considerable periods of time on a diet without the unidentified factor (X) had kidneys which were much heavier than those of their much larger sex-litter mates receiving the factor. In a later publication[5] Zucker and Zucker described high blood non-protein nitrogen and urea levels at the time when the animals showed the most marked signs of deficiency. McGinnis and

[1] C. A. Cary, A. M. Hartman, L. P. Dryden, and G. D. Likely, *Federation Proc.* **5**, 128 (1946).

[2] D. Whitson, H. W. Titus, and H. R. Bird, *Poultry Sci.* **25**, 143 (1946).

[3] T. F. Zucker and L. M. Zucker, *Abstr. Papers 110th Meeting, Am. Chem. Soc.* p. 7B (1946).

[4] C. A. Cary and A. M. Hartman, *Yearbook Agr. 1943–1947, U. S. Dept. Agr.* p. 779 (1947).

[5] L. M. Zucker and T. F. Zucker, *Arch. Biochem.* **16**, 115 (1948).

coworkers[6] stated that blood non-protein nitrogen was "greatly elevated" in deficient chicks receiving the basal diet *plus* either vitamin-free casein or "α protein" but was lowered to control levels when an alcohol-soluble liver fraction was added to the basal diet. Schultze[7] found that acute uremia occurred in newborn rats from mothers on diets containing soybean protein and methionine as the only sources of amino acids. The uremia was prevented by injecting the young subcutaneously with 0.05 γ of vitamin B_{12} soon after birth. It was noted[8] that the blood levels of non-protein nitrogen and of seven individual amino acids were lower in chicks which received a supplement containing vitamin B_{12} than in chicks on a basal diet deficient in the vitamin.

B. VITAMIN B_{12} AND THYROTOXICITY

The feeding of thyroxine or desiccated thyroid in large doses to adult rats has been reported to cause losses of body and ovarian weight, accompanied by anestrus, which can be prevented by high levels of thiamine or yeast.[9] These supplements were reported not to be effective in young rats.[10] The existence of a dietary factor in liver which alleviated the toxicity of thyroid powder for immature rats was noted in 1947 by Ershoff,[11] who found that yeast, wheat germ, casein, and ten synthetic B vitamins were ineffective, and by Betheil and coworkers, who reported that high levels of yeast or moderate levels of dried liver were similarly effective.[12] These findings were used by Robblee and coworkers in studies with chicks.[13] It was found that the thyrotoxic condition was counteracted by supplementing the diet with fish solubles or by injecting anti-pernicious anemia liver extract, and the conclusion was drawn that the addition of either 0.125% desiccated thyroid or 0.02 to 0.03% iodinated casein to the basal diet improved the assay procedure for the unidentified chick growth factor present in fish solubles and liver extract. The use of thyroid-supplemented diets in the assay of liver preparations with rats and mice was adopted by various investigators,[14-16] and rats on such diets were shown to respond

[6] J. McGinnis, P. T. Hsu, and W. D. Graham, *Poultry Sci.* **27**, 674 (1948).
[7] M. O. Schultze, *Proc. Soc. Exptl. Biol. Med.* **72**, 613 (1949).
[8] L. W. Charkey, H. S. Wilgus, A. R. Patton, and F. X. Gassner, *Proc. Soc. Exptl. Biol. Med.* **73**, 21 (1950).
[9] V. A. Drill, R. Overman, and J. H. Leathem, *Endocrinology* **32**, 327 (1943).
[10] B. H. Ershoff, *Endocrinology* **37**, 218 (1945).
[11] B. H. Ershoff, *Proc. Soc. Exptl. Biol. Med.* **64**, 500 (1947).
[12] J. J. Betheil, V. D. Wiebelhaus, and H. A. Lardy, *J. Nutrition* **34**, 431 (1947).
[13] A. R. Robblee, C. A. Nichol, W. W. Cravens, C. A. Elvehjem, and J. G. Halpin, *Proc. Soc. Exptl. Biol. Med.* **67**, 400 (1948).
[14] U. D. Register, W. R. Ruegamer, and C. A. Elvehjem, *J. Biol. Chem.* **177**, 129 (1949).

to vitamin B_{12}.[17-19] However, Ershoff[20] reported that liver residue contained a factor other than vitamin B_{12} which completely counteracted the growth-retarding effect of desiccated thyroid on rats fed purified diets containing casein as the source of protein and sucrose as the source of carbohydrate.

Evidently, the requirement for vitamin B_{12} in rats, mice, and chicks is increased by adding thyroid to the diet. The mechanism of this interaction is unknown. In addition, the hyperthyroidism induced on certain diets may be counteracted by an unidentified factor in liver residue. Recent work by Ershoff indicated[21] that soybean flour, kidney, and crude aureomycin mash could replace the liver residue.

It was suggested by Rupp and coworkers[22] that increased food intake might partly account for the beneficial effects of vitamin B_{12} on hyperthyroid animals, and these investigators studied this point with groups of "force-fed" rats. Their results indicated that vitamin B_{12} could decrease the loss of nitrogen resulting from the catabolic action of thyroxine in force-fed rats on constant food intake.

A sex difference in the response of hyperthyroid rats to vitamin B_{12} was noted by Bolene *et al.*,[23] who also found that maximum responses were not obtained unless various crude materials were added to the diet. Further studies[24] gave some indication that vitamin B_{12} was less effective than a crude liver fraction for normal males and ovariectomized females, but the two supplements produced the same response when fed to castrated males and normal females.

C. VITAMIN B_{12} AND LIVER FUNCTION

The relation of vitamin B_{12} and folic acid to the metabolism of choline and methionine (pp. 432 to 443) raised the possibility that these vitamins might be shown to be involved in the prevention of fatty livers in rats,

[15] D. K. Bosshardt, W. J. Paul, K. O'Doherty, J. W. Huff, and R. H. Barnes, *J. Nutrition* **37**, 21 (1949).

[16] D. V. Frost, H. H. Fricke, and H. C. Spruth, *Proc. Soc. Exptl. Biol. Med.* **72**, 102 (1949).

[17] J. J. Betheil and H. A. Lardy, *J. Nutrition* **37**, 495 (1949).

[18] U. D. Register, V. J. Lewis, and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.* **70**, 167 (1949).

[19] G. A. Emerson, *Proc. Soc. Exptl. Biol. Med.* **70**, 392 (1949).

[20] B. H. Ershoff, *Arch. Biochem.* **28**, 359 (1950).

[21] B. H. Ershoff, Paper presented at Nutrition Symposium, Yale University, Nov. 10, 1950.

[22] J. Rupp, K. E. Pashkis, and A. Cantarow, *Proc. Soc. Exptl. Biol. Med.* **76**, 432 (1951).

[23] C. Bolene, O. B. Ross, and R. MacVicar, *Proc. Soc. Exptl. Biol. Med.* **75**, 610 (1950).

[24] A. B. Watts, O. B. Ross, C. K. Whitehair, and R. MacVicar, *Proc. Soc. Exptl. Biol. Med.* **77**, 624 (1951).

especially on diets which supplied borderline quantities of precursors of choline. Hall and Drill[25] found that either choline or crude liver extract prevented fatty infiltration and fibrosis in the livers of rats fed a diet containing 16% casein and 59% fat. The effects of the liver extract could not be attributed to its choline content.

Another reason for a connection between vitamin B_{12} and liver function was postulated by Stern and coworkers.[26] These workers, noting the relationship of vitamin B_{12} to the formation of desoxyribosides, investigated the effect of vitamin B_{12} upon nucleic acids in the rat as measured by the concentration of basophilia in the liver tissue. It was found that rats which were deficient in vitamin B_{12} showed few or no liver basophilia whereas those which received vitamin B_{12} or liver grew well and had a considerable number of cytoplasmic basophilia in their liver cells. This suggested to Popper and coworkers[27] that vitamin B_{12} might alleviate the hepatic injury due to carbon tetrachloride poisoning, since ribonucleic acid disappears from the cells of the liver in the early stages of carbon tetrachloride intoxication. Administration of 15 γ of vitamin B_{12} per 100 g. of body weight to rats preceding a toxic dose of 0.033 ml. of carbon tetrachloride per 100 g. inhibited the development of histologic changes, the deposition of lipids, and the retention of bromosulfalein. György and Rose[28] also found that vitamin B_{12} had a lipotropic effect in rats when 0.5 γ was fed daily.

Confirmatory evidence of the preventive effects of vitamin B_{12} against carbon tetrachloride poisoning was given by Mushett,[29] who found protection against a single dose, but when both substances were given daily for a period of three weeks, the vitamin failed to prevent the hepatic changes. Hove and Hardin[30] found that either vitamin B_{12} or vitamin E protected against carbon tetrachloride toxicity. The requirement of vitamin B_{12} for protection was considerably higher than for growth.

In studies with dogs[31] a basal diet was used containing 30% extracted peanut meal, 39.5% sucrose, 6% washed casein, 19% lard, 0.1% cystine, vitamins (except B_{12}), and minerals, *plus* choline 0.05%. Severe edema and ascites were developed which could be prevented by adding a concentrate of vitamin B_{12} or by raising the level of choline to 0.30%. Cirrhosis was noted in several dogs on the low-choline diet without vitamin B_{12}.

[25] C. A. Hall and V. A. Drill, *Proc. Soc. Exptl. Biol. Med.* **69**, 3 (1948).

[26] J. R. Stern, M. W. Taylor, and W. C. Russell, *Proc. Soc. Exptl. Biol. Med.* **70**, 551 (1949).

[27] H. Popper, D. Koch-Weter, and P. B. Szanto, *Proc. Soc. Exptl. Biol. Med.* **71**, 688 (1949).

[28] P. György and C. S. Rose, *Proc. Soc. Exptl. Biol. Med.* **73**, 372 (1950).

[29] C. W. Mushett, *Federation Proc.* **9**, 339 (1950).

[30] E. L. Hove and J. O. Hardin, *Proc. Soc. Exptl. Biol. Med.* **77**, 502 (1951).

[31] A. E. Schaefer, D. H. Copeland, and W. D. Salmon, *J. Nutrition* **43**, 201 (1951).

Burns and McKibbin[32] used 19% "vitamin-test" casein in purified diets for puppies, together with 68% carbohydrate, 7% cottonseed oil, 2% cod liver oil, minerals, and vitamins (without choline). The animals grew slowly and in many cases developed fatty livers. The livers of dogs had low total lipid contents in a similar experiment where supplementary choline was fed. Two dogs on this regime were put back on the basal diet and developed signs of liver dysfunction, including high fat content of liver biopsy samples, in 70 to 100 days. Injections of 0.4 mg. of vitamin B_{12} were then given on alternate days, following which there was a reduction in plasma alkaline phosphatase and in liver fat. The authors considered that the diet had produced a deficiency which responded to either vitamin B_{12} or choline.

D. VITAMIN B_{12} AND TYROSINE METABOLISM

Disturbances in tyrosine metabolism have been noted in pernicious anemia,[33] and, since pteroylglutamic acid (PGA) has been related to the oxidation of tyrosine,[34, 35] speculation has arisen that these disturbances might be caused by the PGA deficiency which is associated with pernicious anemia.[36] Since vitamin B_{12}, like PGA, causes hemopoietic remission in pernicious anemia, the urinary phenol fractions were studied in patients during the progress of remission under treatment with vitamin B_{12}.[37] Prompt changes in these fractions were observed following this treatment. A decrease in the ratio of hydroxyphenyl acids to ether-soluble phenols not soluble in $NaHCO_3$ was noted in three of four patients. It is not possible to say whether these changes were caused by vitamin B_{12} *per se* or whether B_{12} had an effect on the metabolism of folic acid which in turn affected the breakdown of tyrosine.

E. SINGLE-CARBON UNITS

The participation of certain sources of a "single-carbon fragment" including formate, formaldehyde, and methanol in a number of biochemical reactions has been studied principally in rats and in tissue preparations derived from them. The use of isotopically tagged compounds has made possible the exploration and extension of this field.

Formate, glycine, and serine are related in the following manner:

$$\dot{C}H_2(NH_2)COOH \rightarrow H\dot{C}OOH + \dot{C}H_2(NH_2)COOH \rightleftharpoons \dot{C}H_2OH\dot{C}H(NH_2)COOH$$

[32] M. M. Burns and J. M. McKibbin, *J. Nutrition* **44**, 487 (1951).

[33] M. E. Swendseid, B. Wandruff, and F. H. Bethell, *J. Lab. Clin. Med.* **32**, 1242 (1947).

[34] G. Rodney, M. E. Swendseid, and A. L. Swanson, *J. Biol. Chem.* **168**, 395 (1947).

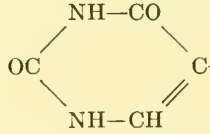
[35] C. W. Woodruff and W. J. Darby, *J. Biol. Chem.* **172**, 851 (1948).

[36] G. Rodney, M. E. Swendsied, and A. L. Swanson, *J. Biol. Chem.* **179**, 19 (1949).

[37] L. D. Abbott, Jr. and G. W. James, III, *J. Lab. Clin. Med.* **35**, 35 (1950).

TABLE I

INTERRELATIONSHIPS INVOLVING THE SINGLE-CARBON UNIT DEMONSTRATED WITH ISOTOPICALLY LABELED COMPOUNDS (FROM SHIVE[39])

Precursors	Products	Biological systems	References
$H—C^{14}OOH$	2,8-C^{14}-Uric acid	Pigeons	40
$H—C^{14}OOH$	C^{14}-Hypoxanthine	Pigeon liver homogenates	41
$H—C^{14}OOH$	2,8-C^{14}-Guanine, C^{14}-adenine	Rats	42
$H—C^{14}OOH$		Rats	43
$H—C^{14}OOH$, $NH_2—CH_2—C^{13}OOH$	$HO—C^{14}H_2—CH(NH_2)C^{13}OOH$	Rats	44
$H—C^{14}OOH$, $NH_2—CH_2—COOH$	$HO—C^{14}H_2—CH(NH_2)—COOH$	Rat liver slices	45
		Rat liver homogenates	38
$H—C^{14}OOH$	$C^{14}H_3—S—CH_2—CH_2—CH(NH_2)—COOH$	Rats, rat liver slices	47
		Rat liver homogenates	38, 48
$H—C^{14}OOH$	$(C^{14}H_3)N^+—CH_2—CH_2OH^a$	Rat liver slices	47
		Rats	49
$H—C^{14}OOH$, $N^{15}H_2—CH_2—COOH$	$(C^{14}H_3)_3N^{15+}—CH—CH_2OH^a$	Rats	50
$H—C^{14}HO$, $NH_2—CH_2—COOH$	$HO—C^{14}H_2—CH(NH_2)—COOH$	Rat liver homogenates	38
			38
$H—C^{14}HO$	$C^{14}H_3—S—CH_2—CH_2—CH(NH_2)—COOH$	Rat liver	38
$H—C^{14}HO$	$(C^{14}H_3)_3N^+—CH_2—CH_2OH^a$	Rats	49
$C^{14}H_3OH$	$(C^{14}H_3)_3N^+—CH_2—CH_2OH^a$	Rats	51
$C^{14}H_3OH$, $N^{15}H_2—CH_2—COOH$	$(C^{14}H_3)_3N^{15+}—CH_2—CH_2OH^a$	Rats	50
$C^{14}H_3OH$	C^{14}-Creatine	Rats	51

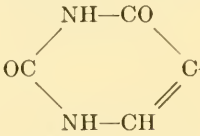
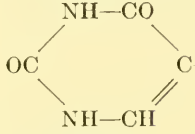
$C^{14}H_3OH$, $NH_2—CH_2—COOH$	$HO—C^{14}H_2—CH(NH_2)—COOH$	Rat liver homogenates	38
$(C^{14}H_3)_3N^+—CH_2—CH_2OH$	$HO—C^{14}H_2—CH(NH_2)—COOH$	Rats	52
$(C^{14}H_3)_3N^+—CH_2—CH_2OH$	$H—C^{14}OOH$, $H—C^{14}HO$	Rat liver slices	48
$(CH^2{}_3)_3N^+—CH_2—CH_2OH$	H^2-Creatine, H^2-creatinine	Rats	53
$CH^2{}_3—S—CH_2—CH_2—CH(NH_2)—COOH$	$(CH^2{}_3)_3N^+—CH_2—CH_2OH$, H^2-creatine	Rats	53
$C^{14}H^2{}_3—S—CH_2—CH_2—CH(NH_2)—COOH$	$(C^{14}H^2{}_3)_3N^+—CH_2—CH_2OH$,[a, b] C^{14}, H^2-creatine	Rats	54
$CH^{14}H_3—S—CH_2—CH_2—CH(NH_2)—COOH$	$H—C^{14}OOH$, $H—C^{14}HO$	Rat liver slices	48
$C^{14}H_3—S—CH_2—CH_2—CH(NH_2)—COOH$	C^{14}-Adenine	Rat liver homogenates	55
$C^{14}H_3—S—CH_2—CH_2—CH(NH_2)—COOH$	C^{14}-Cystine, C^{14}-*p*-bromophenylmercapturic acid[c]	Rats	56
$C^{14}H_3—NH—CH_2—COOH$	$H—C^{14}OOH$, $H—C^{14}HO$, $C^{14}O$	Rat liver homogenates	55
$(C^{14}H_3)_2N—CH_2—COOH$	$H—C^{14}OOH$	Rat liver homogenates	55
$HO—C^{14}H_2—CH(NH_2)—COOH$	$H—C^{14}OOH$, $H—C^{14}HO$	Rat liver slices	38
$HO—C^{14}H_2—CH(NH_2)—COOH$	$(C^{14}H_3)_3N^+—CH_2—CH_2OH$[a, d]	Rats	50, 57
$HO—C^{14}H_2—CH(N^{15}H_2)—COOH$	$(C^{14}H_3)_3N^{15+}—CH_2—C^{14}H_2OH$[e]	Rats	58
$HO—C^{14}H_2—CH(NH_2)—COOH$	 C^{14}-Adenine, C^{14}-guanine	Rats	59
$HO—C^{14}H_2—CH(NH_2)—COOH$	2,8-C^{14}, 7-N^{15}-uric acid	Pigeons	60
$HO—CH_2—CH(N^{15}H_2)—COOH$			
$HO—CH_2—CH(N^{15}H_2)—C^{13}OOH$	$C_6H_5—CO—N^{15}H—CH_2—C^{13}OOH$	Rats, guinea pigs	61
$NH_2—CH_2—C^{13}OOH$	$HO—CH_2—CH(NH_2)—C^{13}OOH$	*Torulopsis utilis*	52
$NH_2—CH_2—C^{14}OOH$	$HO—CH_2—CH(NH_2)—C^{14}OOH$	Rats	63
		Rat liver slices or homogenates	45
		Rat liver homogenates	38

TABLE I—*Continued*

Precursors	Products	Biological systems	References
$NH_2—CH_2—C^{14}OOH$, $N^{15}H_2—CH_2—COOH$	$(CH_3)_3N^{16+}—CH_2—CH_2OH$	Rats	50
$N^{15}H_2—CH_2—C^{14}OOH$	$(CH_3)_3N^{15+}—CH_2—CH_2OH$	Rats	68
$NH_2—CH_2—C^{14}OOH$	C^{14}-Cystine	Rats	63
$NH_2—C^{14}H_2—COOH$	$H—C^{14}OOH$,[f] $H—C^{14}HO$	Rat liver slices	45, 64
$NH_2—C^{14}H_2—COOH$	C^{14}-Serine	Rats	63
		Rat liver homogenates	65
$NH_2—C^{14}H_2—COOH$	$HO—C^{14}H_2—C^{14}H(NH_2)—COOH$	Rats	66
		Rat liver slices	46
			48, 64
$NH_2—C^{14}H_2—COOH$	2,8-C^{14}-Uric acid	Pigeons	67
$NH_2—C^{14}H_2—COOH$	$(C^{14}H_3)_3N^+—CH_2—CH_2OH$[a]	Rats	50
$N^{15}H_2—C^{14}H_2—COOH$	$(C^{14}H_3)_3N^{16}—C^{14}H_2—C^{14}H_2OH$	Rats	58
$NH_2—C^{14}H_2—COOH$		Rats	59
	C^{14}-Adenine, C^{14}-guanine		
$NH_2—C^{14}H_2—COOH$	C^{14}-Cystine, C^{14}-*p*-bromophenylmercapturic acid[c]	Rats	56, 63
$CH_3—CO—C^{14}H$	$HO—C^{14}H_2—CH(NH_2)—COOH$	Rats	68
$CH_3—CO—C^{14}H_3$	$C^{14}H_3—S—CH_2—CH_2—CH(NH_2)—COOH$	Rats	68
$CH_3—CO—C^{14}H_3$	$(C^{14}H_3)_3N^+—CH_2—CH_2OH$	Rats	68

[a] C^{14} also occurs in the ethanolamine moiety, presumably derived from incorporation of C^{14} into serine

[b] C^{14}/H^2 ratio essentially unchanged.

[c] Probably the result of the formation of labeled serine which is converted into cysteine.

[d] Only L-serine utilized. Similarly labeled D-serine does not give rise to labeled choline.

[e] Appreciable amounts of C^{14} also in other carbons of ethanolamine moiety.

[f] Reaction not reversible.

In addition, formate can give rise to the methyl carbon of choline, and the side chain of choline may be derived from serine so that by the administration of α-tagged glycine to a rat it is possible to obtain from the tissues choline with all its carbon atoms labeled. Formaldehyde has been reported to be even more effective than formate in giving rise to the β-carbon of serine.[38] The carbon atoms in the purine ring in the 2 and 8 positions, in the methyl group of methionine, and in the 5-methyl group of thymine have all been shown to be capable of being derived from formate carbon. The experimental work in this field has recently been summarized in tabular form by Shive[39] (Table I). Vitamin B_{12} and folic acid have been implicated as coenzyme-like factors in many of these chemical reactions.

Glycine can give rise to formate in biological systems, and formate can provide the carbon atom for the methyl groups of choline, methionine, and thymine, for the 2 and 8 positions of the purine ring, and for the β-carbon of serine. It therefore follows that any enzyme system which aids in the production of formate from glycine or with the subsequent utilization of formate will be implicated in the biological systhesis of those compounds into which the carbon atom of formate is incorporated. Vitamin B_{12} and *p*-aminobenzoic acid have been shown by Shive[69] to be involved in *E. coli*

[38] I. Siegel and J. Lafaye, *Proc. Soc. Exptl. Biol. Med.* **74**, 620 (1950).
[39] W. Shive, *Vitamins and Hormones* **9**, 110 (1951).
[40] J. M. Buchanan and J. C. Sonne, *J. Biol. Chem.* **166**, 781 (1946).
[41] G. R. Greenberg, *Arch. Biochem.* **19**, 337 (1948).
[42] M. R. Heinrich and D. W. Wilson, *J. Biol. Chem.* **186**, 447 (1950).
[43] J. R. Totter, E. Volkin, and C. E. Carter, Paper presented at 118th Meeting, American Chemical Society, September, 1950.
[44] W. Sakami, *J. Biol. Chem.* **176**, 995 (1948).
[45] P. Siekevitz and D. M. Greenberg, *J. Biol. Chem.* **180**, 845 (1949).
[46] M. E. Coates, J. E. Ford, G. F. Harrison, S. K. Kon, J. W. G. Porter, W. F. J. Cuthbertson, and H. F. Pegler, *Biochem. J.* **49**, lxvii (1951).
[47] A. D. Welch and W. Sakami, *Federation Proc.* **9**, 245 (1950).
[48] P. Siekevitz and D. M. Greenberg, *Federation Proc.* **9**, 227 (1950); *J. Biol. Chem.* **186**, 275 (1950).
[49] V. du Vigneaud, W. G. Verly, and J. E. Wilson, *J. Am. Chem. Soc.* **72**, 2819 (1950).
[50] H. R. V. Arnstein, *Biochem. J.* **47**, xviii (1950).
[51] V. du Vigneaud and W. G. Verly, *J. Am. Chem. Soc.* **72**, 1049 (1950).
[52] W. Sakami, *J. Biol. Chem.* **179**, 495 (1949).
[53] V. du Vigneaud, M. Cohn, J. P. Chandler, J. R. Schenck, and S. Simmonds, *J. Biol. Chem.* **140**, 625 (1941).
[54] E. B. Keller, J. R. Rachele, and V. du Vigneaud, *J. Biol. Chem.* **177**, 733 (1949).
[55] C. G. MacKenzie, *J. Biol. Chem.* **186**, 351 (1950).
[56] J. A. Stekol, K. Weiss, and S. Weiss, *J. Biol. Chem.* **185**, 271 (1950).
[57] S. Jonsson and W. A. Mosher, *J. Am. Chem. Soc.* **72**, 3316 (1950).
[58] H. Weissbach, D. Elwyn, and D. B. Sprinson, *J. Am. Chem. Soc.* **72**, 3316 (1950).
[59] D. Elwyn and D. B. Sprinson, *J. Am. Chem. Soc.* **72**, 3317 (1950).

in the synthesis of methionine, purines, serine, and thymine. Similarly folic acid has been found to be involved in the synthesis of thymine and purines by *L. casei*,[70] and of thymidine by *Le. mesenteroides*,[71] in the conversion of glycine to serine[72] via formate,[73] and of homocystine to methionine.[74] This close interrelationship of vitamin B_{12} and folic acid in biochemical reactions finds its counterpart in the interchangeable effects of these two vitamins in producing a hemopoietic response in pernicious anemia.

The basis of the relationship between vitamin B_{12} and folic acid is a subject for speculation. Shive observed that vitamin B_{12} was able to reverse the toxicity of sulfanilamide for *E. coli* under highly specific conditions. Since *p*-aminobenzoic acid would also reverse this toxicity, Shive[39] suggested that vitamin B_{12} was either involved in the conversion of *p*-aminobenzoic acid to the coenzyme form or it had an independent coenzymic function in the reactions involving the single carbon unit. On the other hand, Davis[75] has discussed the possibility that *p*-aminobenzoic acid may enter the vitamin B_{12} molecule as a building stone. The evidence for this was based on the observation that vitamin B_{12} had a sparing effect on the requirement of certain *E. coli* mutants for *p*-aminobenzoic acid. It was also noted that certain mutants ("quintuple auxotrophs") required tyrosine, phenylalanine, tryptophan, *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid but did not require vitamin B_{12}, perhaps suggesting the structural origin of the benzene ring of vitamin B_{12} from *p*-aminobenzoic acid. The molar requirement of *E. coli* for vitamin B_{12} was found to be 0.02 of its requirement for *p*-aminobenzoic acid.

The discovery that *p*-aminobenzoic acid was present in the molecule of

[60] D. Elwyn and D. B. Sprinson, *J. Biol. Chem.* **184**, 465 (1950).
[61] D. Shemin, *J. Biol. Chem.* **162**, 297 (1946).
[62] G. C. H. Ehrensvard, E. Sperber, E. Saluste, L. Reio, and R. Stjernholm, *J. Biol. Chem.* **169**, 759 (1947).
[63] P. D. Goldsworthy, T. Winnick, and D. M. Greenberg, *J. Biol. Chem.* **180**, 341 (1949).
[64] P. Sickevitz, T. Winnick, and D. M. Greenberg, *Federation Proc.* **8**, 250 (1949).
[65] T. Winnick, I. Moring-Claesson, and D. M. Greenberg, *J. Biol. Chem.* **175**, 127 (1948).
[66] W. Sakami, *J. Biol. Chem.* **178**, 519 (1949).
[67] J. L. Karlsson and H. A. Barker, *J. Biol. Chem.* **177**, 597 (1949).
[68] W. Sakami, *Federation Proc.* **9**, 222 (1950).
[69] W. Shive, Presented at N. Y. Academy of Sciences, Symposium on Antimetabolites, Feb. 11, 1949.
[70] E. E. Snell and H. K. Mitchell, *Proc. Natl. Acad. Sci. U. S.* **27**, 1 (1941).
[71] E. L. R. Stokstad, *J. Biol. Chem.* **139**, 475 (1941).
[72] B. Holland and W. W. Meinke, *J. Biol. Chem.* **178**, 7 (1949).
[73] G. W. E. Plaut, J. J. Betheil, and H. A. Lardy, *J. Biol. Chem.* **184**, 795 (1950).
[74] M. A. Bennett, *J. Biol. Chem.* **187**, 751 (1950).
[75] B. D. Davis, *J. Bacteriol.* **62**, 221 (1951).

pteroylglutamic acid[76] made it seem that the sole function of *p*-aminobenzoic acid might be to serve as a precursor of folic acid, and indeed this seemed possible in the case of *S. faecalis* Ralston, which was found by Lampen and Jones[77] to utilize pteroylglutamic acid as a non-competitive antagonist for sulfanilamide. This indicated that sulfanilamide inhibited this organism solely by blocking the conversion of *p*-aminobenzoic acid to folic acid. However, for many microorganisms, *p*-aminobenzoic acid cannot be replaced by folic acid as a growth factor,[75, 78, 79] thus making it evident that *p*-aminobenzoic acid has functions other than that of serving as a precursor of folic acid. To interpret the existing observations, Shive[39] inclines to the viewpoint that *p*-aminobenzoic acid and folic acid may be the precursors of a single coenzyme and that organisms differ in their ability to utilize the two respective precursors in the formation of the coenzyme. However, this explanation does not appear to be full enough to account for the suggestion by Davis[75] that *p*-aminobenzoic acid may be a precursor of vitamin B_{12}. Again, if one assumes that *p*-aminobenzoic acid and folic acid may both serve in the formation of the same coenzyme and that the utilization of *p*-aminobenzoic acid for this purpose is catalyzed by vitamin B_{12} as follows:

$$p\text{-Aminobenzoic acid} \xrightarrow[B_{12}]{} \text{Coenzyme} \leftarrow \text{Folic acid}$$

it would then be difficult to account for the observation that many species, such as chicks,[80] rats,[74] and human beings,[81] require both vitamin B_{12} and folic acid. A deficiency of folic acid appeared to be intensified by the administration of vitamin B_{12} in the report by Day and coworkers.[81] This would appear to rule out the possibility that vitamin B_{12} and folic acid contribute toward the formation of the same coenzyme. Chicks will develop folic acid deficiency even when vitamin B_{12} and *p*-aminobenzoic acid are both supplied;[80] indeed, there is no coherent evidence that the latter substance plays a role in animal nutrition as a dietary essential or otherwise.

A functional derivative of folic acid, the citrovorum factor (CF), is needed by *Leuconostoc citrovorum* and is more effective than pteroylglutamic acid in reversing the toxicity of certain "folic acid antagonists."[82, 83] It is

[76] R. B. Angier, J. H. Boothe, B. L. Hutchings, J. H. Mowat, J. Semb, E. L. R. Stokstad, Y. SubbaRow, C. W. Waller, D. B. Cosulich, M. J. Fahrenbach, M. E. Hultquist, E. Kuh, E. H. Northey, D. R. Seeger, J. P. Sickels, and J. M. Smith, Jr., *Science* **103**, 667 (1946).

[77] J. O. Lampen and M. J. Jones, *J. Biol. Chem.* **164**, 485 (1946).

[78] J. O. Lampen, M. J. Jones, and R. R. Roepke, *J. Biol. Chem.* **180**, 423 (1949).

[79] N. S. Cutts and C. Rainbow, *Nature* **164**, 234 (1949).

[80] E. L. R. Stokstad and T. H. Jukes, *Proc. Soc. Exptl. Biol. Med.* **62**, 112 (1946).

[81] L. A. Day, B. E. Hall, and G. L. Pease, *Proc. Staff Meet. Mayo Clinic* **24**, 119 (1949).

[82] H. E. Sauberlich and C. A. Baumann, *J. Biol. Chem.* **176**, 165 (1948).

tempting but at present fruitless to speculate that CF might be the postulated coenzyme formed from folic acid and *p*-aminobenzoic acid as suggested above. There is no evidence that CF will replace *p*-aminobenzoic acid as a bacterial growth factor. Vitamin B_{12} will not replace CF as a growth factor for *Le. citrovorum*[84] or as a detoxifying agent for the folic acid antagonists,[85] and in turn CF will not replace vitamin B_{12} as a growth factor for *L. leichmannii*[86] or chicks.[87] Ascorbic acid rather than vitamin B_{12} appears to be involved in the urinary excretion of increased amounts of CF following the administration of folic acid.[88, 89]

Separate roles in certain microorganisms for vitamin B_{12} and CF, respectively, in the formation of desoxyribosides and in their methylation were suggested by Jukes and coworkers.[86] Any of several purine and pyrimidine desoxyribosides could replace vitamin B_{12} as a growth factor for *L. leichmannii*, but only thymine desoxyriboside would replace CF for growth of *Le. citrovorum*.

F. RELATION OF VITAMIN B_{12} TO THE METABOLISM OF CHOLINE AND METHIONINE

1. In Chicks

A borderline deficiency of methionine was recognized as being the first limiting amino acid deficiency in soybean meal in diets for chicks, even when the soybean meal was suitably heated so as to make its protein content nutritionally available.[90-92] Sardine or herring fish meal were also noted to improve growth markedly when added to such diets,[93-95] and the suggestion was made by Bird and Mattingly that there might exist in fish meal a

[83] H. E. Sauberlich, *Federation Proc.* **8**, 247 (1949).
[84] H. P. Broquist, E. L. R. Stokstad, C. E. Hoffman, M. Belt, andd T. H. Jukes, *Proc. Soc. Exptl. Biol. Med.* **71**, 549 (1949).
[85] H. E. Skipper, J. B. Chapman, and M. Bell, *Cancer Research* **11**, 161 (1951).
[86] T. H. Jukes, H. P. Broquist, and E. L. R. Stokstad, *Arch. Biochem.* **26**, 157 (1950).
[87] T. H. Jukes and coworkers, Unpublished investigations.
[88] C. A. Nichol and A. D. Welch, *Proc. Soc. Exptl. Biol. Med.* **74**, 52 (1950).
[89] H. P. Broquist, E. L. R. Stokstad, and T. H. Jukes, *J. Lab. Clin. Med.* **38**, 95 (1951).
[90] J. W. Hayward and F. H. Hafner, *Poultry Sci.* **20**, 139 (1941).
[91] H. J. Almquist, E. Mecchi, F. H. Kratzer, and C. R. Grau, *J. Nutrition* **24**, 385 (1942).
[92] J. A. Marvel, C. W. Carrick, R. E. Roberts, and S. M. Hauge, *Poultry Sci.* **23**, 294 (1944).
[93] J. B. Christiansen, H. J. Deobald, J. G. Halpin, and E. B. Hart, *Poultry Sci.* **19**, 18 (1940).
[94] J. S. Carver and R. J. Evans, *U. S. Egg Poultry Mag.* **49**, 468 (1943).
[95] J. C. Hammond and H. W. Titus, *Poultry Sci.* **23**, 49 (1944).

substance, other than methionine, choline, or cystine, capable of performing in part the same biological functions as methionine.[96]

Unidentified factors in sardine fish meal and yeast were shown to influence the growth response of chicks to methionine by Patton and coworkers in 1946.[97] Some of their data are shown in Table II. These findings showed that a growth response in chicks was produced by the addition of methionine to a corn-soybean meal diet. A greater growth response was produced when, instead of methionine, a small amount of fish meal was added to the basal diet. The amount of methionine in the fish meal corresponded to a quantity which was too small to produce a growth response, for no growth effect was produced by adding an equivalent amount of methionine in the

TABLE II

Effect of Methionine and Sardine Fish Meal on Growth of Chicks When Added to a Corn-Soybean Meal Diet[97]

Additions to basal diet		
Sardine fish meal, %	α-Methionine, %	Body weight at 4 weeks, g.
None	None	202
None	0.15	252
None	0.30	252
2.0	None	304
2.0	0.15	305
2.0	0.30	300
4.0	None	307
4.0	0.15	305
4.0	0.30	283

form of vitamin-free casein. Furthermore, when fish meal was present in the diet, the addition of methionine did not produce a growth response. These findings indicated that fish meal contained an unidentified factor which diminished the requirement of chicks for methionine under the experimental conditions encountered. The authors showed that it was possible to prepare a water-soluble fraction from sardine fish meal which was fully effective at a level of 0.06%. They also showed that with purified diets consisting of starch, casein, and gelatin no improvement was produced by fish meal. It is now known that casein often contains vitamin B_{12}, which would account for the lack of a response to fish meal under such conditions. It appears certain that the factor studied in these investigations

[96] H. R. Bird and J. P. Mattingly, *Poultry Sci.* **24**, 29 (1945).

[97] A. R. Patton, J. P. Marvel, H. G. Petering, and J. Waddell, *J. Nutrition* **31**, 485 (1946).

was vitamin B_{12}, which is present in fish meal and has been shown to have an effect on the response to methionine corresponding to that encountered by Patton and coworkers. In other experiments (Table III) a similar effect was noted for yeast extract, and in this case later evidence indicates that folic acid may have been the factor involved, since yeast does not supply effective amounts of vitamin B_{12}. Thus vitamin B_{12} and folic acid both have been shown to be needed for the biochemical reactions which are concerned with the metabolism of the group of compounds, including methionine, supplying "labile methyl" groups. Patton *et al.* concluded with the following significant comment ". . . fish meal increases chick growth on a corn-soybean diet by supplying some factor, known or unknown, whose requirement has been augmented, or created, by the presence of corn and/or soybean oil meal in the diet. This would explain the failure to discover any

TABLE III

Effect of Methionine and Yeast Extract on the Growth of Chicks When Added to a Basal Diet of Corn and Soybean Meal Supplemented with Vitamins and Choline[97]

Additions to basal diet		
Yeast extract, %	Methionine, %	Body weight at 4 weeks, g.
None	None	251
None	0.30	293
0.50	None	286
0.50	0.30	289

such factor using the purified diet. . . . The factor would be of great practical importance."

A relation between vitamin B_{12} and methionine was described by Shive[69] in January 1949. He reported that the crystalline vitamin could function interchangeably with methionine in enabling the growth of *Escherichia coli* to take place on a medium containing sulfanilamide. One part of vitamin B_{12} was as active as 300,000 parts of methionine. Analogous findings were reported by Davis and Mingioli,[98] who isolated mutants of *E. coli* which were unable to grow in the absence of sulfanilamide unless vitamin B_{12} or methionine was provided, under which conditions the mutants produced a substance which appeared to be homocysteine. Homocysteine was ineffective, thus indicating a catalytic role for vitamin B_{12} in the transformation of homocysteine to methionine.

It was reported by Carrick and coworkers that either choline, methionine,[99] or betaine,[100] when added to a basal diet consisting principally of

[98] B. D. Davis and E. S. Mingioli, *J. Bacteriol.* **60**, 17 (1950).

[99] R. W. Gerry, C. W. Carrick, and S. M. Hauge, *Poultry Sci.* **27**, 161 (1948).

corn and soybean meal, would increase the rate of growth of chicks. These findings were followed by a report by Gillis and Norris,[101] who also found that either choline or betaine would produce added growth in chicks on a diet of cereals and soybean meal. The addition of 0.16% of "liver paste" containing vitamin B_{12} activity was even more effective than choline or betaine in improving growth, and if choline or betaine were added in addition to the liver paste, no further improvement in growth was obtained. At about this time, Schaefer and coworkers reported that concentrates containing vitamin B_{12} had a sparing effect on the choline requirement of chicks; at 8 weeks the weight gain of chicks receiving 0.2% of choline plus a source of vitamin B_{12} was equal to that of chicks receiving 0.6% of choline without vitamin B_{12}.[102] Gillis and Norris[103] then showed that vitamin B_{12} behaved in a manner similar to liver paste in replacing the growth-promoting effect of choline or betaine under the conditions previously reported by them.

The effects of vitamin B_{12} on the response to choline and methionine by chicks were studied by Jukes and Stokstad.[104] Vitamin B_{12}-deficient chicks were fed a purified diet deficient in choline and containing 20% casein and 0.4% cystine with added vitamins including folic acid. Either choline or vitamin B_{12} markedly increased the growth of chicks on this diet, but maximum growth was not obtained unless both supplements were given, nor was perosis prevented unless 0.15% of choline was added to the diet. The addition of vitamin B_{12} hastened the onset of perosis. Growth was slower at a level of 0.6% choline than at a level of 0.3% choline, whether or not vitamin B_{12} was administered. A diet containing ground peas, gelatin, and glucose supplemented with choline, tryptophan, and vitamins including folic acid was used for the production of methionine deficiency in vitamin B_{12}-deficient chicks. Growth was increased by either methionine or vitamin B_{12} but was subnormal unless both supplements were given.

Studies with chicks on a diet deficient in both vitamin B_{12} and methionine were reported by Jukes and coworkers.[105] The diet consisted of glucose, alcohol-extracted soybean protein, cystine, dimethylaminoethanol, minerals, and vitamins. Homocystine did not promote the growth of vitamin B_{12}-deficient chicks on this diet even when betaine was added, but the chicks responded to the addition of methionine. When supplemented with

[100] O. H. Mishler, C. W. Carrick, and S. M. Hauge, *Poultry Sci.* **28**, 24 (1949).

[101] M. B. Gillis and L. C. Norris, *J. Biol. Chem.* **179**, 487 (1949).

[102] A. E. Schaefer, W. D. Salmon, and D. R. Strength, *Federation Proc.* **8**, 395 (1949); *Proc. Soc. Exptl. Biol. Med.* **71**, 202 (1949).

[103] M. B. Gillis and L. C. Norris, *Poultry Sci.* **28**, 749 (1949).

[104] T. H. Jukes and E. L. R. Stokstad, *Abstr. Papers 116th Meeting, Am. Chem. Soc.* p. 40A (1949); *J. Nutrition* **43**, 459 (1951).

[105] T. H. Jukes, E. L. R. Stokstad, and H. P. Broquist, *Arch. Biochem.* **25**, 453 (1950).

vitamin B_{12}, the chicks responded to methionine or homocystine. These results were interpreted as indicating that vitamin B_{12} is involved in the transformation of homocystine to methionine in chicks.

Interrelationships between vitamin B_{12} and various precursors of choline were studied in chicks by Schaefer *et al.*[106] The basal diet consisted of extracted peanut meal, sucrose, extracted casein, lard, cystine, minerals, and vitamins. Some results are shown in Table IV; vitamin B_{12} where included was added at a level of 30 γ per kilogram of diet. The absence of

TABLE IV

EFFECT OF CHOLINE AND RELATED COMPOUNDS ON 4-WEEK WEIGHT GAINS, PEROSIS, AND MORTALITY IN CHICKS[102]

Compound tested in grams per kilogram of diet	Vitamin B_{12}	Average weight gain, g.	Perosis, %	Mortality, %
0.5 choline chloride	+	122	60	0
1.0 choline chloride	+	207	0	0
19.8 betaine hydrochloride	+	95	100	10
0.5 choline chloride + 0.55 betaine hydrochloride	+	127	10	0
0.5 choline chloride + 0.55 betaine hydrochloride	+	166	40	9
1.61 methylaminoethanol	+	55		0
0.54 methylaminoethanol + 3.3 betaine hydrochloride	0	100	25	50
0.54 methylaminoethanol + 3.3 betaine hydrochloride	+	206	0	0
0.54 methylaminoethanol + 2.1 DL-methionine	0	86	50	66
0.54 methylaminoethanol + 2.1 DL-methionine	+	195	5	0

appropriate controls without vitamin B_{12} makes it difficult to interpret the data completely; however, the authors conclude that vitamin B_{12} appeared to be involved in the synthesis of choline from methylaminoethanol in the presence of methionine or betaine as judged by the ability of these combinations to replace choline when vitamin B_{12} was added to the diet. It was also noted that even when vitamin B_{12} was added aminoethanol plus betaine could not serve as precursors of choline as judged by growth response and the prevention of perosis.

In 1950 Briggs and coworkers[107] used non-depleted chicks fed 'practical-type" diets consisting principally of corn and soybean meal. They found

[106] A. E. Schaefer, W. D. Salmon, and D. R. Strength, *J. Nutrition* **44**, 305 (1951).
[107] G. M. Briggs, E. G. Hill, and M. J. Giles, *Poultry Sci.* **29**, 723 (1950).

that methionine could replace the vitamin B_{12} requirement of their chicks. However, this does not apply to depleted chicks fed high soybean-meal diets, for an acute deficiency was observed in 1949 by Stokstad and coworkers[108] in chicks receiving a diet containing 70% soybean meal with the addition of 0.3% methionine and 0.2% choline chloride. Similar results were reported by Ott,[109] who used a diet containing 70% soybean meal with 0.9% methionine and 0.2% choline chloride and found the requirement of vitamin B_{12} for chicks to be 27 γ per kilogram of diet.

TABLE V

Diets Used in the Study of the Growth of Chicks as Affected by "Methylating Agents"[110]

	Diet		
	1	2	3
Glucose ("Cerelose")	61.5 g.	61.5 g.	—
Soybean protein	25 g.	25 g.	14 g.
Ground dried split peas	—	—	70 g.
Gelatin	—	—	8 g.
Calcium gluconate	5 g.	5 g.	—
Corn oil + vitamins A, D, and E	3 g.	3 g.	1 g.
Bone ash	2 g.	2 g.	3 g.
Salt mixture	2 g.	2 g.	—
Sodium chloride	—	—	0.5 g.
L-Cystine	0.3 g.	0.3 g.	—
DL-Tryptophan	—	—	0.2 g.
Dimethylaminoethanol	0.2 g.	—	—
Manganese sulfate	—	—	25 g.
Aureomycin HCl	2 mg.	2 mg.	2 mg.
Pteroylglutamic acid	—	0.5 mg.	0.2 g.
Vitamin mixture[a]	+	+	+

[a] Inositol 100 mg., niacinamide 5 mg., calcium pantothenate 5 mg., thiamine HCl 1 mg., riboflavin 1 mg., pyridoxine HCl 1 mg., vitamin K compound 0.5 mg., biotin 0.02 mg.

The effects of various combinations of vitamin B_{12}, folic acid, homocystine, choline, betaine, and methionine were studied in vitamin B_{12}-depleted chicks on deficient diets by Jukes and Stokstad.[110] The basal diets used are shown in Table V. With diet 1, it was found that any of the following substances would produce a growth response: folic acid, vitamin B_{12}, betaine, choline, or methionine. Some typical results are shown in Table VI. The data show that the response to methionine was affected very little

[108] E. L. R. Stokstad, T. H. Jukes, J. V. Pierce, A. C. Page, Jr., and A. L. Franklin, *J. Biol. Chem.* **180**, 647 (1949).

[109] W. H. Ott, *Poultry Sci.* **30**, 86 (1951).

[110] T. H. Jukes and E. L. R. Stokstad, *J. Nutrition* (In press.)

by vitamin B_{12} or folic acid. There was no growth response to homocystine unless vitamin B_{12} was also added; the responses to mixtures of homocystine and betaine or homocystine and choline in the absence of vitamin B_{12} was no greater than the responses to betaine or choline when added singly. However, when vitamin B_{12} was supplied, there was a response to homocystine. The growth-promoting effect of folic acid was markedly affected by the other supplements; a sparing action of folic acid on the requirement for choline is illustrated by the following data:

TABLE VI

WEIGHTS OF CHICKS AT 25 DAYS ON PURIFIED DIET 1 (GLUCOSE + 25% SOYBEAN PROTEIN) WITH VARIOUS SUPPLEMENTS

Vitamin addition per kilograms of diet	Additions per kilogram of diet						
	None	6 g. DL-homocystine	2 g. betaine	2 g. choline	Homocystine + betaine	Homocystine + choline	2 g. DL-methionine
None	75	74	108	141	119	140	176
5 mg. PGA	92	88		155	144	151	202
50 γ B_{12}	182	242		233	296	315	312
B_{12} + PGA	255	321		255	338	324	349

Additions	Weight at 25 days
Vitamin B_{12}	182
Vitamin B_{12} + folic acid	225
Vitamin B_{12} + choline	233
Vitamin B_{12} + folic acid + choline	255
Vitamin B_{12} + homocystine	242
Vitamin B_{12} + homocystine + folic acid	321
Vitamin B_{12} + homocystine + choline	325
Vitamin B_{12} + homocystine + folic acid + choline	324

These data can be explained by assuming that folic acid in the presence of vitamin B_{12} is needed for the methylation of dimethylaminoethanol to form choline. When preformed choline was supplied, the requirement for folic acid for this purpose disappeared.

Using diet 2 which contained added folic acid but not dimethylaminoethanol, it was again found that no response was produced by homocystine unless vitamin B_{12} was added, but methionine produced responses regardless of the presence or absence of vitamin B_{12}. The results are shown in Tables VII and VIII. The leg-bone deformity termed "perosis" was aggravated by vitamin B_{12} in the absence of choline, confirming an earlier observation.[104] When diet 3, which was more deficient in methionine than diets 1 and 2, was used, the effect of vitamin B_{12} on the utilization of homocystine was even more marked and is illustrated in Fig. 5. If, however,

the ground peas in the basal diet were extracted with hot alcohol, the growth response to homocystine in the presence of vitamin B_{12} largely disappeared, but it could be restored by adding choline to the diet as shown in Table VIII. This leads to the conclusion that choline was an effective methylating agent for homocystine under the conditions of these experi-

TABLE VII

Weight of Chicks at 25 Days on Purified Diet 2 with Various Supplements; Dimethylaminoethanol Omitted; PGA Added to Basal Diet

First addition per kilogram of diet	Supplemental addition				
	Choline			Betaine	
	None	0.2%	0.4%	0.2%	0.4%
None	77	135	149	107	129
Homocystine	74	130		90	
Vitamin B_{12}	165	276	263	191	163
B_{12} + homocystine	193	311		191	
Methionine	153	178		166	
B_{12} + methionine	210	251		236	

TABLE VIII

Effect of Various Supplements on Perosis in Chicks on Diet 2

Additions to diet	Perosis score[a] at 25 days with				
	Choline			Betaine	
	None	0.2%	0.4%	0.2%	0.4%
None	3	1	3	17	8
Homocystine	7	1		15	
Methionine	7	5		7	
Vitamin B_{12}	28	4	5	29	32
Vitamin B_{12}	34	3		32	
Vitamin B_{12}	25	2		21	

[a] Maximum possible score = 40.

ments, and that glycine and serine, which were undoubtedly present in substantial quantities in the basal diet, were relatively ineffective.

2. In Rats

The growth of rats in response to various sulfur amino acids was studied by White and Beach.[111] They used a diet containing starch, hydrogenated cottonseed oil, arachin, salt mixture, and cod liver oil. The rats received daily supplements of 100 mg. of rice bran extract and 100 mg. of crude

[111] A. White and E. F. Beach, *J. Biol. Chem.* **122**, 219 (1937).

liver extract powder. The basal diet was incapable of supporting growth, and very little improvement was obtained by adding cystine, but the addition of either methionine or homocystine enabled growth to take place. The presence of liver extract in the diet used in these experiments is of much significance when viewed in the light of later findings.

It was found[112] that rats usually lost weight when fed an amino acid mixture without methionine but containing homocystine. Rice bran extract, 12.5 mg. daily, was the only "crude" B-complex supplement added to the diet. Rice bran, like all cereal products, is low in folic acid and

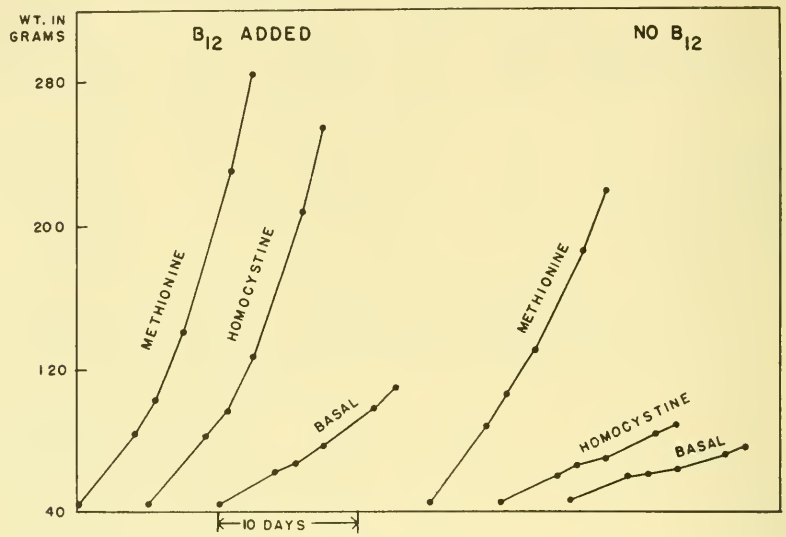


FIG. 5. Growth of chicks on a methionine-deficient diet with various supplements in the presence and absence of vitamin B_{12}.

vitamin B_{12}. The addition of choline to the diet or the substitution of methionine for homocystine enabled growth to take place. However, a few animals on the basal diet grew slowly without added choline, and the authors speculated as to the possibility of "refection" being an explanation for this apparent anomaly. The involvement of vitamin-like factors in the utilization of homocystine by rats was studied by Bennett and Toennies,[113] who showed that crude liver extract supplied unidentified factors which enabled rats to grow on a "labile-methyl-free" diet containing homocystine and succinylsulfathiazole. No growth was obtained when a more refined and concentrated extract was used. This latter extract was shown elsewhere

[112] V. du Vigneaud, J. P. Chandler, A. W. Moyer, and D. M. Reppel, *J. Biol. Chem.* **131**, 57 (1939).

[113] M. A. Bennett and G. Toennies, *J. Biol. Chem.* **163**, 235 (1946).

to be almost completely free from folic acid.[80, 114] Later it was reported by Bennett[74] that both vitamin B_{12} and folic acid were needed to produce growth in rats on such a diet. The growth was slow, 0.8 to 1.0 g. daily. The addition of succinylsulfathiazole had evidently prevented the rats from obtaining a supply of folic acid from their intestinal flora. When vitamin B_{12} and folic acid were added in the experiments by Bennett, it may be conjectured that certain of the dietary amino acids, perhaps glycine and serine, were able to provide the necessary precursors for the methylation of homocystine. It is important to note that methionine could bring about the resumption of growth even when vitamin B_{12} and folic acid were not added.[113]

The findings of Bennett were confirmed by Stekol and Weiss,[115] who included folic acid in their basal diet and emphasized the role of vitamin B_{12}, whereas the experiments of Bennett were carried out under conditions of folic acid deficiency which drew attention to the well-known role of the intestinal bacteria in supplying folic acid. The basal diet used by Stekol and Weiss contained dextrin, sucrose, fat, amino acids, minerals, and vitamins. Rats 30 days or older were able to grow on such diets in the absence of methionine and choline if vitamin B_{12} and homocystine (or homocysteine) were added. If vitamin B_{12} was omitted, they did not grow with homocystine, but they grew at a suboptimal rate if methionine was added, or if homocystine and choline were added. A relation between vitamin B_{12} and the formation of methionine was indicated by the observations of Oginsky,[116] who found that liver homogenates from vitamin B_{12}-deficient rats showed a lowered ability to form methionine as compared with homogenates from animals receiving vitamin B_{12} when homocystine was added with choline or betaine to the homogenates. In another report,[117] homogenates of livers of rats deficient in folic acid or B_{12} or both were studied. It was reported that B_{12} deficiency was associated with a lowered capacity for the synthesis of methionine from either choline or betaine and homocysteine, confirming the observations by Oginsky,[116] whereas folic acid-deficient homogenates showed a reduced ability to synthesize cysteine from homocysteine and serine, presumably via cystathionine.

Studies of the biological production and utilization of formate in the tissues of rats were shown to involve "labile methyl" groups. By the use of isotopically labeled compounds it has been found that glycine could give rise to formate which could combine with a second molecule of glycine to form serine. Furthermore, the carbon atom of formate, the β-carbon atom

[114] G. W. Clark, *Am. J. Med. Sci.* **209**, 520 (1945).
[115] J. A. Stekol and K. W. Weiss, *J. Biol. Chem.* **186**, 343 (1950).
[116] E. L. Oginsky, *Arch. Biochem.* **26**, 327 (1950).
[117] J. Stekol, K. W. Weiss, and S. Weiss, *Federation Proc.* **10**, 252 (1951).

of serine, or the α-carbon atom of glycine was shown to serve as a source of the methyl carbon atom of choline or methionine. The extent to which glycine and serine can replace choline for the methylation of homocystine in the rat is at present ill-defined. A report by Arnstein and Neuberger[118] indicates that rats on a "labile-methyl-free" diet containing homocysteine and vitamin B_{12} grew more rapidly in the presence of choline than in its absence.

Dinning and coworkers[119] investigated the requirement of the rat for "methylating" compounds in the treatment of leucopenia induced by feeding a low-choline, low-methionine diet containing folic acid to rats. They found that the requirement could be satisfied by betaine in the presence of vitamin B_{12} or by methionine alone. For further studies, folic acid was omitted from the basal diet and it was found that methionine was effective in the absence of both vitamin B_{12} and folic acid, whereas choline or betaine were effective only in the presence of these two vitamins.[120]

It was suggested[121, 122] that vitamin B_{12} was a cofactor in the transformation of glycine into serine. However, a study of the radioactivity of the β-carbon atom of serine following the administration of α-C^{14}-glycine to rats did not indicate that vitamin B_{12} deficiency depressed this transformation.[123] The study showed that the deficiency lowered the rate of conversion of glycine, serine, and formate into the CH_3 group of methionine and into all the carbons of choline. In pair-fed rats, vitamin B_{12} deficiency slowed the passage of the carbon of α-labeled glycine into (1) the CH_3 group of methionine in the viscera but not in the carcass and (2) the trimethylamine group of choline, but not the total molecule. The difference between the results with full-fed rats and those with paired-fed rats makes it evident that the interpretation of such results is difficult.

The effects of deficiencies of vitamin B_{12} and folic acid on the formation of glutathione in the intact rat were studied by Anderson and Stekol,[124] who found that the deficiencies did not affect the incorporation of glycine or cystine, respectively, into the glycine or cysteine components of glutathione, but that either deficiency decreased the conversion of the α-carbon of glycine into the cysteine and glutamic acid portions of the glutathione molecule.

[118] H. R. V. Arnstein and A. Neuberger, *Biochem. J.* **48**, ii (1951).
[119] J. S. Dinning, C. K. Keith, and P. L. Day, *J. Biol. Chem.* **189**, 515 (1951).
[120] J. S. Dinning, C. K. Keith, and P. L. Day, *J. Nutrition* **43**, 525 (1951).
[121] H. R. V. Arnstein and A. Neuberger, *Biochem. J.* **50**, xxxviii, 1952.
[122] J. A. Stekol, S. Weiss, and K. W. Weiss, *Arch. Biochem.* **36**, 5, 1952.
[123] H. R. V. Arnstein and A. Neuberger, *Biochem. J.* **55**, 259, 1953.
[124] E. I. Anderson and J. A. Stekol, *J. Biol. Chem.* **202**, 611, 1953.

The methionine requirement of chicks for growth was found to be increased by deficiencies of either vitamin B_{12} or folic acid.[125]

G. VITAMIN B_{12} AND TUMORS

Woolley[126] has observed that spontaneous mammary tumors of mice may possess the ability to synthesize vitamin B_{12}. This ability of the cancerous tissue would be a distinct difference from normal mouse tissue and would constitute an important clue to the etiology and the treatment of at least this particular type of tumor. It was noted that the nursing young from tumor-bearing mothers gained normally on a vitamin B_{12}-deficient ration, whereas the young from non-tumorous mothers gained at a slower rate recognized to be due to vitamin B_{12} deficiency. The fact that only spontaneous tumors appeared to synthesize vitamin B_{12}, whereas transplanted tumors did not, somewhat complicates the interpretation of the observed phenomenon.

V. Specificity of Action

DONALD E. WOLF and KARL FOLKERS

In discussing the specificity of action of vitamin B_{12}, some limitations must be made, since the title could include a broad review of all functions of the vitamin in human, animal, and microbial metabolism. The treatment presented here is intended to give a general survey of the subject and is therefore not complete.

A. ANIMAL AND MICROBIOLOGICAL METABOLISM

Vitamin B_{12} has been found to affect the phosphorus metabolism of *Lactobacillus leichmannii*.[1] When this organism was grown in a medium containing radioactive phosphorus, vitamin B_{12} was found to increase the uptake of phosphorus in the desoxyribonucleic acid fraction of the cell to a more pronounced extent than in the total cell or in the acid-insoluble portion. This observation is in accord with the concept previously suggested[2] that vitamin B_{12} is involved in nucleic acid synthesis.

Extensive experiments have been carried out to demonstrate the effect

[125] T. H. Jukes, Paper presented at 124th Meeting of the American Chemical Society, Chicago, September, 1953.

[126] D. W. Woolley, *Proc. Natl. Acad. Sci. U. S.* **39**, 6, 1953.

[1] I. Z. Roberts, R. B. Roberts, and P. H. Abelson, *J. Bacteriol.* **58**, 709 (1949).

[2] W. Shive, J. M. Ravel, and R. E. Eakin, *J. Am. Chem. Soc.* **70**, 2614 (1948).

of vitamin B_{12} on egg production and hatchability in the domestic fowl. With a sucrose-soybean protein diet low in vitamin B_{12}, hens showed a decrease in egg production. Hatchability of the eggs from hens fed this vitamin B_{12}-deficient diet also decreased to zero in 3 to 6 weeks.[3] Addition of APF concentrates to the diet improved the egg production and hatchability but failed to produce the normal hatchability of hens on a practical all-mash diet. Incubation of eggs from hens on a vitamin B_{12}-deficient diet resulted in embryonic mortality reaching a peak at the seventeenth day.[4] The most characteristic symptoms of the deficiency present in the embryos was myoatrophy of the leg and a malposition, "head between thighs." Other anomalies included hemorrhages of the embryo, allantois, and perosis. Injection of vitamin B_{12} into eggs laid by hens on a vitamin B_{12}-deficient diet improved the hatchability and lowered mortality among the chicks which hatched.[5]

The effect of vitamin B_{12} on the anemia produced in chicks by injection of phenylhydrazine hydrochloride has been studied.[6] Neither liver extract nor vitamin B_{12} alone influenced the rate of hemoglobin formation, but in combination with folic acid both showed activity in bringing about a more rapid regeneration of hemoglobin. A deficiency of vitamin B_{12} and lysine in the diet of cockerels resulted in failure of morphological differentiation of testicular tissue accompanied by a decrease in comb growth.[7] Supplementation with vitamin B_{12} and lysine produced greater testicular growth and differentiation over that of the normal controls.

Hyperthyroid rats require a growth factor present in liver, fish solubles, and tomatoes.[8] Vitamin B_{12} is a growth factor in this deficiency, but folic acid is not effective in growth promotion of such animals.

Vitamin B_{12} has been found to have an effect on the metabolism of amino acids, enhancing their utilization for building fixed tissues.[9] It plays a fundamental role, affecting the capacity of the normal mammal to utilize protein;[10] animal deficiencies in vitamin B_{12} may even be fatal in the presence of high levels of protein.

[3] O. Olcese, J. R. Couch, and C. M. Lyman, *J. Nutrition* **41**, 73 (1950).

[4] O. Olcese, J. R. Couch, J. H. Quisenberry, and P. B. Pearson, *J. Nutrition* **41**, 423 (1950).

[5] R. J. Lillie, M. W. Olson, and H. R. Bird, *Proc. Soc. Exptl. Biol. Med.* **72**, 598 (1949).

[6] C. A. Nichol, A. E. Harper, and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.* **71**, 34 (1949).

[7] F. X. Gassner, A. R. Patton, H. S. Wilgus, and L. W. Charkey, *Proc. Soc. Exptl. Biol. Med.* **75**, 630 (1950).

[8] J. J. Bethell and H. A. Lardy, *J. Nutrition* **37**, 495 (1949).

[9] L. W. Charkey, H. S. Wilgus, A. R. Patton, and F. X. Gassner, *Proc. Soc. Exptl. Biol. Med.* **73**, 21 (1950).

[10] A. M. Hartman, L. P. Dryden, and C. A. Cary, *Arch. Biochem.* **23**, 165 (1949).

B. HUMAN METABOLISM

Early work on vitamin B_{12} was prompted by a desire to find the anti-pernicious anemia factor. Many fractions from liver were tested for activity on addisonian pernicious anemia cases. The first successful use of crystalline vitamin B_{12} in the treatment of this disease was reported by West in 1948.[11] Vitamin B_{12} seems to be effective for the relief of all symptoms of addisonian pernicious anemia. Cases with pernicious anemia in relapse show a rapid hematological response similar to that produced by liver extract with an almost immediate increase in reticulocyte count and gradual rise in hemoglobin and erythrocyte regeneration.[11-15] Erythrocyte regeneration from megaloblastic to normoblastic types of cells may occur in 48 to 72 hours when relatively large amounts of vitamin B_{12} are administered.[13] Improvement in strength, mental alertness, and appetite is characteristic, with weight gains and disappearance of glossitis.[14] Vitamin B_{12} appears to be quite specific for relief of combined degeneration of the spinal cord and involvement of the central nervous system which often accompany addisonian pernicious anemia.[14-18] Lingual manifestations of pernicious anemia appear to respond well to vitamin B_{12} therapy.[19]

Administration of vitamin B_{12} to a series of twenty-five premature infants did not effect better growth as compared to a series of untreated premature infants.[20] In two cases of megaloblastic anemia of infancy, administration of vitamin B_{12} produced a hemopoietic response.[21]

Vitamin B_{12} is effective in a number of other hematological diseases where anemia is a primary symptom. Nutritional macrocytic anemia,[22] tropical[23] and non-tropical sprue,[22] and tropical macrocytic anemia[24] all responded with prompt or striking clinical improvement when treatment with vitamin B_{12} was initiated.

A number of investigators have carried out clinical trials of the various

[11] R. West, *Science* **107**, 398 (1948).
[12] E. Jones, W. J. Darby, and J. R. Totter, *Blood* **4**, 827 (1949).
[13] B. E. Hall, and D. C. Campbell, *Proc. Staff Meet. Mayo Clinic* **23**, 584 (1948).
[14] B. E. Hall and D. C. Campbell, *Proc. Staff. Meet. Mayo Clinic* **23**, 591 (1948).
[15] B. E. Hall and D. C. Campbell, *J. Lab. Clin. Med.* **33**, 1646 (1948).
[16] T. D. Spies, R. E. Stone, G. Garcia Lopez, F. Milanes, T. Aramburu, and R. Lopez Toca, *Postgrad. Med.* **4**, 89 (1948).
[17] J. Kaufmann and A. Cooperberg, *Can. Med. Assoc. J.* **60**, 552 (1949).
[18] T. D. Spies, R. E. Stone, S. Kartus, and T. Aramburu, *Southern Med. J.* **41**, 1030 (1948).
[19] J. F. Schieve and R. W. Rundles, *J. Lab. Clin. Med.* **34**, 439 (1949).
[20] D. F. Downing, *Science* **112**, 181 (1950).
[21] A. Z. McPherson, U. Jonsson, and R. W. Rundles, *J. Pediat.* **34**, 529 (1949).
[22] T. D. Spies, R. E. Stone, and T. Aramburu, *Southern Med. J.* **41**, 522 (1948).
[23] T. D. Spies, G. Garcia Lopez, F. Milanes, R. Lopez Toca, and B. Culver, *Southern Med. J.* **41**, 523 (1948).
[24] J. C. Patel, *Brit. Med. J.* **1948**, 934.

modifications of cyanocobalamin. Although the reports are not all conclusive, it seems evident that all the substances so far tested possess very nearly the same activity as vitamin B_{12} in the clinical tests conducted. Quantitative estimates in tests of this nature are very difficult. Hydroxocobalamin (vitamins B_{12a} or B_{12b}) has been found to promote clinical improvement and produce positive hemopoietic responses in cases with addisonian pernicious anemia, tropical sprue, non-tropical sprue, nutritional macrocytic anemia, and with one case of megaloblastic anemia of infancy.[25, 26] Sulfatocobalamin, cyanatocobalamin, and the vitamin B_{12}-hydrogen sulfide reaction product have been found potent in addisonian pernicious anemia, nutritional macrocytic anemia, and sprue.[27] Vitamins B_{12c} and B_{12d} (B_{12a}) have both been tested clinically in a number of cases of addisonian pernicious anemia and subacute combined degeneration of the spinal cord with the result that no difference could be observed between the activities of these compounds and that of vitamin B_{12}.[28, 29]

Extensive experiments have been carried out on the relationship of vitamin B_{12}, folic acid, thymine, and uracil in persons with pernicious anemia and related megaloblastic anemias.[30, 31] It has been concluded from this work, in agreement with previously cited experiments on microbial metabolism,[1, 2] that both folic acid and vitamin B_{12} are involved or are essential to the formation of nucleic acid and nucleoprotein. The suggestion has been made that the megaloblast common to pernicious anemia and related macrocytic anemias is a primitive erythroblast with an abnormality in the metabolism of nucleoprotein due to deficiency of vitamin B_{12}, folic acid, and possibly other chemical activators.[30]

VI. Biogenesis

DONALD E. WOLF and KARL FOLKERS

Production of vitamin B_{12} is by biosynthesis using fermentation processes. Investigations have been conducted to determine means of increasing

[25] E. A. Kaczka, R. G. Denkewalter, A. Holland, and K. Folkers, *J. Am. Chem. Soc.* **73**, 335 (1951).

[26] T. D. Spies, R. E. Stone, M. B. Koch, H. M. Grant, and M. M. Moore, *Southern Med. J.* **43**, 50 (1950).

[27] E. A. Kaczka, D. E. Wolf, F. A. Kuehl, and K. Folkers, *J. Am. Chem. Soc.* **73**, 3569 (1951).

[28] C. C. Ungley and H. Campbell, *Brit. Med. J.* **I**, 152 (1951).

[29] J. N. M. Chalmers, *Brit. Med. J.* **I**, 161 (1951).

[30] R. W. Vilter, D. Horrigan, J. F. Mueller, T. Jarrold, C. F. Vilter, V. Hawkins and A. Seaman, *Blood* **5**, 695 (1950).

[31] D. D. Woods, *J. Pharm. Pharmacol.* **2**, 537 (1950); C. C. Ungley, *ibid.* **2**, 540.

the yield of the vitamin. The most striking discovery was the effect of the addition of cobalt to the fermentation medium in the biosynthesis of LLD-active substances by *Streptomyces griseus* and a large number of other vitamin B_{12}-producing microorganisms.[1] Addition of one or two parts per million of cobalt ion produced optimum production of LLD-active substances. The stimulation due to added cobalt ion has been put to practical use in the formation of vitamin B_{12} containing cobalt 60.[2] By addition of cobalt 60 as the nitrate of specific activity 1.800 μc. per milligram to the nutrient medium, it was possible to obtain vitamin B_{12} with a specific activity of $\simeq 67$ μc. per milligram.[2]

Addition of 1,2-dimethyl-4,5-diaminobenzene to bacterial cultures of *Bacillus megatherium* stimulated the production of vitamin B_{12} and also riboflavin.[3] These results are not surprising; in both vitamins, the diamine may be considered as a degradation product and a precursor.[4, 5] Addition of 1,2-dichloro-4,5-diaminobenzene to similar bacterial cultures brought about an inhibition in the production of vitamin B_{12} and riboflavin when the dichloride was present in an insufficient quantity to inhibit growth of the organism.[3]

The activity of choline chloride has been tested on fourteen strains of microorganisms isolated from poultry house litter. The vitamin B_{12} production of twelve strains was reduced and that of two strains was improved by addition of choline chloride to the medium.[6]

Investigations of the vitamin B_{12} content of the intestinal tract of various animals and man has given some evidence on the biogenesis of the vitamin. Sheep rumen has been found to be a fairly rich source of vitamin B_{12}.[7] About one-half of the cobalt ion fed to sheep was excreted in organically bound form in the feces. Biological assay of the butanol extract of feces indicated the presence of large amounts of vitamin B_{12}.[8] Only two types of bacteria, *Bacillus subtilis* and a corynebacterium, were found in fresh pig stomach mucosa and in a commercial preparation of this tissue.[9] These produce vitamin B_{12} and control bacterial growth in the mucosa. Vitamin B_{12} has been found at various levels in the alimentary canal of man and

[1] D. Hendlin and M. L. Ruger, *Science* **111**, 541 (1950).
[2] C. Rosenblum and D. T. Woodbury, *Science* **113**, 215 (1951); L. Chaiet, C. Rosenblum, and D. T. Woodbury, *ibid.* **111**, 601 (1950).
[3] D. W. Woolley, *Proc. Soc. Exptl. Biol. Med.* **75**, 745 (1950).
[4] N. G. Brink and K. Folkers, *J. Am. Chem. Soc.* **71**, 2951 (1949).
[5] N. G. Brink and K. Folkers, *J. Am. Chem. Soc.* **72**, 4442 (1950).
[6] E. R. Halbrook, F. Cords, A. R. Winter, and T. S. Sutton, *J. Nutrition* **41**, 555 (1950).
[7] V. J. Lewis, U. D. Register, H. T. Thompson, and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.* **72**, 479 (1949).
[8] P. H. Abelson and H. H. Darby, *Science* **110**, 566 (1949).
[9] B. Noer, *Lancet* **259**, 706 (1950).

animals, being greatest below the ileocecal valve.[10] It is suggested that in man the normal requirement of vitamin B_{12} is met by bacterial synthesis in the colon.[10]

Various tissues have been examined for vitamin B_{12}. Beef samples have been found to contain twice as much vitamin B_{12} as normal pork samples.[11] Of the various organs investigated, the kidney has been found to contain the greatest amount of vitamin B_{12}.[12]

VII. Estimation

THOMAS H. JUKES and WILLIAM L. WILLIAMS

A. PHYSICAL

The ultraviolet and visible absorption spectra of the cobalamins may be used in measuring their concentration in solutions which are free from interfering substances. Values for the extinction coefficients of vitamins B_{12} and B_{12b} have been reported in the literature. Vitamin B_{12} has a sharp peak at 361 mμ,[1] $E^{1\%}_{1\,cm.} = 204$, and a broader peak in the visible region at 550 mμ,[2] $E^{1\%}_{1\,cm.} = 63$. These maxima and values are not appreciably altered by changes in the pH of the solution. Vitamin B_{12b} has similar maxima at 351 mμ, $E^{1\%}_{1\,cm.} = 167$, and 525 mμ, $E^{1\%}_{1\,cm.} = 57$.[3] The values apply only to acid or neutral solutions, for in alkaline solution these maxima of vitamin B_{12b} undergo a bathochromic shift.[4] Other $E^{1\%}_{1\,cm.}$ values for absorption maxima have been reported as follows: for vitamin B_{12} at 362 mμ, 194; at 552 mμ, 59; for vitamin B_{12b} at 351 mμ, 165; at 527 mμ, 56.[5]

The name vitamin B_{12a} was proposed for a compound with specific physical and biological properties which was prepared by catalytic hydrogenation of vitamin B_{12} followed by oxidation with atmospheric oxygen.[6] The

[10] W. J. C. Dyke, H. G. Hind, D. Riding, and G. E. Shaw, *Lancet* **258**, 486 (1950).

[11] U. D. Register, U. J. Lewis, H. T. Thompson, and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.* **70**, 167 (1949).

[12] U. J. Lewis, U. D. Register, and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.* **71**, 508 (1949).

[1] B. Ellis, V. Petrow, and G. F. Snook, *J. Pharm. Pharmacol.* **1**, 60 (1949).

[2] N. G. Brink, D. E. Wolf, E. A. Kaczka, E. L. Rickes, F. R. Koniuszy, T. R. Wood, and K. Folkers, *J. Am. Chem. Soc.* **71**, 1854 (1949).

[3] J. V. Pierce, A. C. Page, Jr., E. L. R. Stokstad, and T. H. Jukes, *J. Am. Chem. Soc.* **72**, 2615 (1950).

[4] J. A. Brockman, Jr., J. V. Pierce, E. L. R. Stokstad, H. P. Broquist, and T. H. Jukes, *J. Am. Chem. Soc.* **72**, 1942 (1950).

[5] W. G. Jackson, G. B. Whitfield, W. H. DeVries, H. A. Nelson, and J. S. Evans, *J. Am. Chem. Soc.* **73**, 337 (1951).

[6] E. A. Kaczka, D. E. Wolf, and K. Folkers, *J. Am. Chem. Soc.* **71**, 1514 (1949).

properties differed from those encountered in a crystalline substance obtained from *Streptomyces aureofaciens*, which was given the name vitamin B_{12b}.[7] Later investigations[4, 8] showed that subsequent attempts to prepare vitamin B_{12a} resulted in the isolation of a substance with the properties of vitamin B_{12b} rather than those described for vitamin B_{12a}. Of much interest was the observation that a band at 315 mμ in the ultraviolet absorption spectrum, which is a prominent characteristic of vitamin B_{12a},[6] tended to disappear when the aqueous solution stood at room temperature. Cooley *et al.*[9] have discussed the possibility that vitamin B_{12b} may be dehydrated with a simultaneous redistribution of charge to give vitamin B_{12a}.

In addition to cyanide, hydroxyl, and nitrite, various other anions may be coordinated in the cobalamin molecule to give a series of compounds with differing absorption spectra. It may thus be seen that the measurement of the vitamins of this group by means of their absorption spectra is difficult to carry out with mixtures of the cobalamins.

B. CHEMICAL

Chemical methods for the determination of small quantities of vitamin B_{12} have been studied by Boxer and Rickards. Their first method[10] was based upon hydrolysis of the vitamin to yield 5,6-dimethylbenzimidazole which was benzoylated to 4,5-dimethyldibenzoyl-*o*-phenylenediamine. This compound was then treated with concentrated sulfuric acid to form 4,5-dimethyl-*o*-phenylenediamine, which was determined colorimetrically or fluorometrically. The procedure was as follows: vitamin B_{12} was hydrolyzed with 6 *N* HCl at 150° for 18 hours to liberate 5,6-dimethylbenzimidazole. Treatment with benzoyl chloride produced 4,5-dimethyldibenzoyl-*o*-phenylenediamine in the Schotten-Baumann reaction, following which the excess benzoyl chloride was converted to sodium benzoate with alkali. The diamine was extracted with chloroform which was removed by evaporation, and the residue was treated with concentrated H_2SO_4 at 150°. For the colorimetric reaction, the sulfuric acid solution of diamine was partially neutralized and treated with acetylacetone to produce 2,4,7,8-tetramethyl-1,5-benzyldiazepine, which has a purple color sensitive enough to determine 10 γ of original benzimidazole. A more sensitive procedure was the fluorometric determination of *o*-phenylenediamine by condensation with alloxan to form the fluorescent compound 6,7-dimethylalloxazine which was ex-

[7] J. V. Pierce, A. C. Page, Jr., E. L. R. Stokstad, and T. H. Jukes, *J. Am. Chem Soc.* **71**, 2953 (1949).

[8] E. A. Kaczka, R. G. Denkewalter, A. Holland, and K. Folkers, *J. Am. Chem. Soc.* **73**, 335 (1951).

[9] G. Cooley, B. Ellis, V. Petrow, G. H. Beaven, E. R. Holiday, and E. A. Johnson, *J. Pharm. Pharmacol.* **3**, 607 (1951).

[10] G. E. Boxer and J. C. Rickards, *Arch. Biochem.* **29**, 75 (1950).

tracted with chloroform, transferred to aqueous alkali, and the fluorescence measured in a Farrand fluorometer. Under these conditions it was possible to determine the benzimidazole over a range of 10 to 100 mγ. Riboflavin did not interfere with the determination. This method does not differentiate vitamin B_{12} from other substances, including cobalamins and degradation products, which contain 5,6-dimethylbenzimidazole.

A more specific method for the determination of vitamin B_{12}, outlined subsequently by Boxer and Rickards, depended upon the determination of cyanide which was liberated from vitamin B_{12} by photolysis with monochromatic light.[11] This procedure was reported to be sufficiently sensitive to determine 1 part of cyanide in 10 parts of solution with a precision of ±1.5%. Hydrogen cyanide was removed by aeration and determined by developing a color reaction with chloramine-T phosphate reagent and pyrazolone-pyridine reagent. The experimental procedure was as follows: Stock solutions were prepared of:

1. Sodium hydroxide, 0.1 *N*.
2. Sodium dihydrogen phosphate, 1.0 *M*.
3. Ceric sulfate, 0.05 *N*, in 1 *N* sulfuric acid.
4. Saturated solution of silver sulfate in 20% sulfuric acid. An excess of solid silver sulfate should always be suspended in the solution.
5. Aqueous solution of chloramine-T (recrystallized), 0.25%. This keeps indefinitely in the refrigerator.
6. Saturated aqueous solution of 3-methyl-1-phenyl-5-pyrazolone (recrystallized). A stock solution is kept with excess solid in a brown bottle and is filtered as needed. This keeps indefinitely.
7. Solution of bis(3-methyl-1-phenyl-5-pyrazolone) in pyridine (reagent grade). This solution is prepared every second day and is kept in a freezing compartment. The solution is discarded when visibly colored.

Reagents Prepared from Stock Solution Prior to Color Development. (a) *Chloramine-T phosphate reagent.* To 1 part of 0.25% chloramine-T is added 3 parts of 1 *M* dihydrogen phosphate. Chloramine-T separates as a colloidal suspension. This reagent can be used as long as chloramine-T does not separate in clumps (usually 15 to 30 minutes). (b) *Pyrazolone-pyridine reagent.* Five parts of a saturated aqueous solution of methylphenylpyrazolone is mixed with 1 part of the solution of bismethylphenylpyrazolone in pyridine. This reagent should be protected from the direct sunlight and discarded whenever a pink color appears (60 to 90 minutes).

Aeration Equipment. A simple aeration train was used throughout. The reaction vessel used for volumes up to 25 ml. was a 25 × 200-mm. test tube; for volumes up to 100 ml., a 38 × 200-mm. test tube; and for volumes up to 500 ml., a 1-l. Florence flask. The reaction vessels were closed with

[11] G. E. Boxer and J. C. Rickards, *Arch. Biochem.* **30**, 372, 392 (1951).

two-hole rubber stoppers fitted with gas inlet and outlet tubes. The final trap which contained the alkali for the collection of the hydrogen cyanide was an 18 × 150-mm. test tube closed by a two-hole rubber stopper The inlet tube reached to the very bottom of the tube to ensure complete immersion in the small volume of alkali. The collection tubes were coated with silicone Dri-film (G.E. No. 9987) applied in the manner described by Doyle and Omoto.[12] The silicone coating prevents continuous film formation and loss of solution through the outlet tube when a rapid stream of gas is bubbled through the alkali. Whenever traps were necessary to remove interfering volatile material, 25 × 200-mm. test tubes were used, fitted exactly like the reaction vessel for a 25-ml. volume. Connections between the various members of the gas train were made with tight-fitting gum rubber tubing. Up to eight individual setups were connected in series and swept by one stream of nitrogen of measured flow rate. The nitrogen, before entering the first reaction vessel, was passed through three traps containing, respectively 20 ml. of acid ceric sulfate, 20 ml. of acid silver sulfate, and 20 ml. of 0.1 *N* NaOH, and finally through an empty trap.

Separation of Cyanide from Thiocyanate. The solution in the reaction vessel, irrespective of the volume used, contained between 0.005 and 0.4 γ of cyanide. The pH of the solution was adjusted to 5 or less. Aeration at this pH separates hydrogen cyanide quantitatively from even 10,000 times as much thiocyanate, provided that no oxidizing agent is present in the solution. In all the biological fluids tested in the subsequent papers, the reduction-oxidation potential was so poised that oxidation of thiocyanate to cyanide did not occur. Whenever there is suspicion that oxidation of thiocyanate can occur, it can be prevented with certainty by the addition of some hypophosphite to the solution.

Collection of Cyanide in Alkali. The hydrogen cyanide swept out of the reaction vessel was in all cases collected in 1 ml. of 0.1 *N* NaOH contained in the 18 × 150-mm. tube.

Separation of Hydrogen Cyanide from Volatile Interfering Materials. Many substrates contain materials which are swept over with the hydrogen cyanide. These interfering materials are prevented from reaching the collection tube by placing the following series of traps between the reaction vessel and the collection tube: (a) an empty tube, (b) a tube containing 20 ml. of 0.5 *N* ceric sulfate in 1.0 *N* sulfuric acid, (c) a tube containing 20 % sulfuric acid saturated with silver sulfate and a few crystals of excess silver sulfate, and (d) a tube containing 20 ml. of 0.1 *N* sulfuric acid.

Whenever these traps are used, 15 minutes must be added to the transfer time. The solutions in the traps should be renewed whenever the ceric ion color fades visibly or the silver trap shows a brownish-black discoloration.

[12] W. L. Doyle and J. H. Omoto, *Anal. Chem.* **22**, 603 (1950).

Colorimetric Determination. The collection tube containing 0.005 to 0.4 γ of cyanide in 1 ml. of 0.1 *N* NaOH is thoroughly chilled in ice. Two-tenths milliliter of chloramine-T–phosphate reagent is added, and the contents are mixed by drawing them once or twice partially up the inlet tube by suction. The tube is replaced in the ice bath, and, after about 2 minutes, 3 ml. of the pyrazolone-pyridine reagent is added and the contents mixed again. The tubes are removed from the ice bath and left at the desired temperature for full color development. The color, once fully developed, is stable for at least 2 hours. The color intensity is measured in a Coleman Model 14 spectrophotometer at 620 mμ, using a PC-5 filter in front of the photocell. Beer's law is obeyed for the range from 0.005 to 0.40 γ of cyanide with a standard deviation of ±1.5%. Precise readings are obtained with the square cuvettes of 13-mm. depth for the range of 0.02 to 0.40 γ, and with the cylindrical cuvette of 50-mm. depth and 3.5-ml. capacity for the range of 0.005 to 0.10 γ of cyanide. The optical density of the blank for the whole procedure (including aeration) was identical with that of distilled water within the limit of errors.

The method was adapted to the determination of total cobalamins by transforming these substances to cyanocobalamin by treatment with cyanide. This was accomplished by adding a three- to fivefold molar excess of freshly prepared aqueous potassium cyanide and holding the solution in the dark for 3 hours at pH 5 or below. The excess cyanide was then removed by aeration in the dark, following which the sample was illuminated and "bound" cyanide was split off from cyanocobalamin by photolysis. The difference between the values for vitamin B_{12} before and after the treatment with cyanide was due to the presence of cobalamins other than cyanocobalamin which were capable of binding cyanide and releasing it on illumination. Data were presented describing the assay of vitamin B_{12} in liver concentrate and other natural materials, and the results were compared with the microbiological procedures.

C. BIOLOGICAL

1. Assay with Chicks

Various modifications of all-vegetable diets are used in the assay of vitamin B_{12} with chicks. The use of high levels of soybean meal in the diet, as described by the Beltsville group,[13] has gained wide acceptance. It is necessary for satisfactory results to use chicks hatched from eggs laid by hens which are on a diet low in vitamin B_{12} because the "carry-over" of this vitamin is pronounced. Furthermore, the hens must be restrained from obtaining appreciable amounts of vitamin B_{12} from the litter on the floor of the hen house.

[13] M. Rubin and H. R. Bird, *J. Nutrition* **34**, 233 (1947).

The following procedure may be used in the biological assay with chicks. The breeding flock are placed on diet 1 (Table IX) at 6 months of age. The birds are kept in a laying house on a sugar-cane litter which is changed weekly, and the eggs laid by these pullets are incubated to serve as a source

TABLE IX

Composition of Basal Diets

Ingredient	Diet 1 (hens)	Diet 2 (chicks)
	g.	g.
Soybean meal, solvent process	30	70
Yellow corn meal	57.8	23.1
Alfalfa meal	5	
Bone meal	4.2	
Calcium carbonate	2.3	2.0
Salt mixture[a]		1.7
Sodium chloride	0.7	0.8
Vitamin mixture in glucose (see below)	1.0	1.0
Corn oil + vitamins A, D, and E[b]		1.0
Vitamins A and D feeding oil	0.2	
Methionine (DL)		0.3
Choline chloride		0.2
Vitamin Mixture	mg.	mg.
Thiamine HCl	1	1
Riboflavin	5	1
Niacinamide	1	5
Pyridoxine	5	1
Calcium pantothenate	1	5
Pteroylglutamic acid	1	0.2
1-Acetoxy-2-methyl-4-naphthyl sodium phosphate		0.5
Biotin		0.02
Glucose	To 1 g.	To 1 g.

[a] The salt mixture is composed of the following in grams: bone ash 1000; K_2HPO_4 200, KH_2PO_4 225, $MgSO_4$ 125, $MnSO_4$ (anhydrous) 40, ferric citrate 25, $CuSO_4 \cdot 5H_2O$ 1.0, KI 0.3, Zn acetate 0.7, $Al_2(SO_4)_3 \cdot 18H_2O$ 0.8, cobalt acetate 0.2, Ni carbonate 0.1.

[b] Vitamin A acetate, 1500 U.S.P. units; vitamin D_3 (Delsterol) 200 A.O.A.C. units; mixed tocopherols, 34 mg. dissolved in 1 g. of corn oil (Mazola).

of chicks, no eggs being used until after the hens have been on diet 1 for at least 2 weeks. If the hatchability of the eggs drops below 60%, 0.5% fish meal is added to diet 1. The chicks are placed in electrically-heated battery brooders with ¾-in. mesh wire floors in an air-conditioned room at 28° and are fed the experimental diets immediately. Diet 2 (Table IX) is used as the deficient basal diet, and duplicate groups of eight to twelve chicks are usually used for each experimental diet. The assay period is 25 days, and the average weights obtained at the end of this period are used

in constructing a standard response curve to vitamin B_{12}, from which the potencies of the unknown samples are calculated.

2. Assay with Rats

Rats were used for the assay of factor X in natural materials by Cary and Hartman and coworkers in their pioneering investigations which were discussed on p. 421. These workers drew attention to the necessity of depleting the maternal stores of vitamin B_{12} so that young rats could be obtained which were sufficiently depleted to be used for the assay which was carried out by observing their growth rate. The investigations of Zucker and Zucker (p. 421) followed similar lines. The degree of depletion encountered in litters of weanling rats was observed to vary with different mothers. Consequently, it was important to subdivide the litters in assembling experimental groups of young rats for the assay.

The addition of desiccated thyroid or iodinated casein has been used by various investigators to increase the deficiency of vitamin B_{12} in young rats with a view to improving the assay.[14] One procedure was as follows: Male weanling rats (Sprague-Dawley) weighing between 40 and 50 g. were used. They were fed a basal diet of the following composition, supplemented with vitamins A and D fed independently:

Whole ground yellow corn	46.35 g.
Commercial soybean meal	46.35 g.
Corn oil (Mazola)	5 g.
Cystine	0.3 g.
$CaHPO_4$	0.92 g.
$CaCO_3$	0.6 g.
Iodized sodium chloride	0.44 g.
$MnSO_4 \cdot 4H_2O$	0.04 g.
Thiamine	0.3 mg.
Riboflavin	0.3 mg.
Niacin	2 mg.
Pyridoxine	0.2 mg.
Pantothenic acid	2 mg.
Folic acid	0.025 mg.
Biotin	0.01 mg.
Inositol	10 mg.
Choline	100 mg.
p-Aminobenzoic acid	25 mg.

To the basal diet was added 0.06 to 0.1 % of iodinated casein. Five rats were used in each group, and the gain in weight was measured over a 2-

[14] U. D. Register, W. R. Ruegamer, and C. A. Elvehjem, *J. Biol. Chem.* **177**, 129 (1949).

to 4-week period. The rates of gain were compared with that obtained on measured doses of vitamin B_{12}.[15]

A procedure for the assay of vitamin B_{12} with rats was described by Frost and coworkers.[16] The diet had the following composition:

Purified casein	18	g./100 g.
Cystine	0.2	
Dextrin	49	
Salt mixture No. 1 (U.S.P.)	4	
Cellu-flour	2	
Agar	1.5	
Primex	5	
Cod liver oil	1	
Choline	0.1	
Sulfaguanidine	0.5	
Protomone (iodinated casein)	0.05	
Riboflavin	3	mg./100 g.
Thiamine HCl	3	
Inositol	20	
Niacin	3	
Pyridoxine HCl	5	
Biotin	0.01	
Folic acid	0.01	
Calcium pantothenate	5	
p-Aminobenzoic acid	5	
Menadione	0.5	

Weanling rats of both sexes were placed on the basal diet for a depletion period of 7 to 14 days, following which six rats were used in each assay group. The average weight gain during the 2-week assay period was used for the assay of vitamin B_{12}. The authors noted that mortality was high on the basal diet during the assay period and that death was generally preceded by the appearance of bloody encrustations about the nose and paws. Vitamin B_{12} prevented these signs.

D. MICROBIOLOGICAL

Shorb reported as early as January 1947 that *Lactobacillus lactis* Dorner (8000) required an unidentified factor which was named the LLD factor[17] and which was present in refined liver extracts. Later Shorb demonstrated[18] that the LLD activity of refined liver extracts closely paralleled the U.S.P.

[15] U. J. Lewis, U. D. Register, and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.* **71**, 509 (1949).

[16] D. V. Frost, H. H. Fricke, and H. C. Spruth, *Proc. Soc. Exptl. Biol. Med.* **72**, 102 (1949).

[17] M. S. Shorb, *J. Bacteriol.* **53**, 669 (1947).

[18] M. S. Shorb, *J. Biol. Chem.* **169**, 455 (1947).

units of liver extracts used for treatment of pernicious anemia. In 1948[19] Shorb tested crystalline vitamin B_{12} and found that it was responsible for the LLD activity of liver extracts. At this time the *L. lactis* Dorner assay for vitamin B_{12} was subject to a great deal of variation. For example, a 60% increase in the response to vitamin B_{12} as compared to liver extract was apparent by simply extending the time of incubation. This organism required the presence of clarified tomato juice (TJ factor)[17-19] which was later found by Caswell *et al.*[20] to be replaceable by a combination of fumaric acid and sodium ethyl oxalacetate in an amino acid medium containing DL-alanine. Shorb demonstrated (personal communication cited in ref. 21) that D-alanine and tomato juice were interchangeable if oxalacetate was in the medium. The factor as it exists in tomato juice is pyridoxamine phosphate.[21] Assays for vitamin B_{12} with *L. lactis* Dorner are further complicated by the requirement of the organism for carbon dioxide[22] When carbon dioxide is present the requirement for vitamin B_{12} is eliminated by anaerobic conditions obtained by the addition of reducing substances to the medium.[23] Shive *et al.*[24] early reported that ascorbic acid added aseptically may eliminate the requirement of *L. lactis* Dorner for vitamin B_{12}. Thymidine and other desoxyribosides stimulate growth of *L. lactis* Dorner and other test organisms of the lactic acid bacteria group under circumstances in which growth is also promoted by vitamin B_{12}, as shown by Shive *et al.*[24] and Wright *et al.*[25] Both these groups of investigators report that an emulsifying agent, Tween 80, is also required for satisfactory growth. According to Shorb and Briggs,[26] *L. lactis* Dorner readily undergoes dissociation on the usual yeast extract-glucose transfer medium, resulting in cultures which do not require vitamin B_{12} or which respond erratically. However, the vitamin B_{12} requirement is relatively stable if the transfer medium consists of skim milk, tomato juice, and yeast extract. Because of the many difficulties attending the use of *L. lactis*, this organism is not generally used in the conventional tube-assay technique. For routine assays *L. lactis* Dorner is used rather widely in cup-plate procedures[27] utilizing solid media.

[19] M. S. Shorb, *Science* **107**, 397 (1948).
[20] M. C. Caswell, L. K. Koditschek, and D. Hendlin, *J. Biol. Chem.* **180**, 125 (1949).
[21] D. Hendlin, M. C. Caswell, V. J. Peters, and T. R. Wood, *J. Biol. Chem.* **186**, 647 (1950).
[22] L. K. Koditschek, D. Hendlin, and H. B. Woodruff, *J. Biol. Chem.* **179**, 1093 (1949).
[23] R. D. Greene, A. J. Brook, and R. B. McCormack, *J. Biol. Chem.* **178**, 199 (1949).
[24] W. Shive, J. M. Ravel, and R. E. Eakin, *J. Am. Chem. Soc.* **70**, 2614 (1948).
[25] L. D. Wright, H. R. Skeggs, and J. W. Huff, *J. Biol. Chem.* **175**, 475 (1948).
[26] M. S. Shorb and G. M. Briggs, *J. Biol. Chem.* **176**, 1463 (1948).
[27] A. L. Bacharach and W. F. J. Cuthbertson, *Analyst* **73**, 334 (1948).

1. Tube Methods for Assay of Vitamin B_{12}

a. Lactobacillus lactis Dorner (8000)

There are three distinctly different procedures for measurement of microbial growth due to vitamin B_{12}. The first is the usual test-tube method in which growth is measured by turbidity readings or titration of acid produced. The newer cup-plate methods already mentioned depend on measurements of the circular areas of growth about a metal cylinder or depression in the agar containing the sample. The third method is the pad technique in which a small volume of the sample extract is pipetted on a small paper pad after which the pad is placed on the solidified test medium. The pad technique has been used with notable success in the assay of vitamin B_{12}, pyridoxine, inositol, biotin, and protogen, a growth factor for protozoans and lactobacilli.[28] *L. lactis* Dorner has been compared with strains of *L. leichmannii* as to differences in response to vitamin B_{12} and vitamin B_{12a} and to crude concentrates of vitamin B_{12} such as liver extracts.[29, 30] Hendlin and Soars[29] found good agreement between assays with *L. lactis* Dorner and *Euglena gracilis* whereas the values obtained with *L. leichmannii* (4797) were significantly lower. No reducing agents were added to the medium. For *L. lactis* Dorner, vitamin B_{12a} autoclaved in the medium was 70 % as active as vitamin B_{12} whereas with *L. leichmannii* (4797) for liver concentrates were approximately one-third of the values obtained with *L. lactis* Dorner. The addition of 0.01 % or 0.02 % thioglycollic acid to the *L. leichmannii* (4797) medium brought the assay values for liver extract and vitamin B_{12a} obtained with this organism up to those obtained with *L. lactis* Dorner.

Cooperman *et al.*[30] compared assay results obtained with a *L. lactis* Dorner (8000) tube method and with the recently adopted U.S.P. method[31] in which *L. leichmannii* 313 (7830) is used. The values for purified and crude liver extracts and for concentrates of fermentation origin obtained with *L. lactis* Dorner were consistently less than one half the values obtained with *L. leichmannii* 313. This discrepancy could be eliminated by adding 0.25 γ of KCN per milliliter to the *L. lactis* Dorner medium, thus increasing the values to those obtained with *L. leichmannii* 313. The *L. lactis* Dorner medium did not contain reducing agents. With *L. leichmannii* 313 a sufficiently high level of KCN (0.25 γ per milliliter) was interchangeable with reducing agents in increasing the assay values over those obtained in the

[28] W. L. Williams, R. G. Esposito, and J. V. Pierce, *Federation Proc.* **11**, 458, 1952.
[29] D. Hendlin and M. H. Soars, *J. Biol. Chem.* **188**, 603 (1951).
[30] J. M. Cooperman, R. Drucker, and B. Tabenkin, *J. Biol. Chem.* **191**, 135 (1951).
[31] U. S. Pharmacopeia, 14th revision, 3rd Supplement, p. 15, 1951.

absence of reducing agents. Vitamin B_{12b} showed about 30 % of the activity of vitamin B_{12} in the *L. lactis* Dorner assay, but the two forms showed equal activity upon the addition of KCN.

Since the *L. lactis* Dorner tube method used by Cooperman *et al.*[30] appears to represent a practical and useful method utilizing this organism, it will be described in some detail. The basal medium used was essentially that of Caswell *et al.*[20] except that several of the pure amino acids were omitted and the amount of acid-hydrolyzed casein doubled. Tween 80 and ammonium acetate were also included.

The detailed composition of the basal medium is given in Table X.

Culture and Inoculum. The stock culture of *L. lactis* Dorner (8000) was transferred weekly on the liver-tryptone agar of Nymon and Gortner.[32]

TABLE X

Composition of Double-Strength Basal Medium

HCl-hydrolyzed casein	1.0 g.	Salts A	2.5 ml.
Glucose	5.0 g.	Salts C	2.5 ml.
Sodium acetate (anhydrous)	3.0 g.	Tween 80	25 mg.
L-Cystine	100 mg.	Calcium pantothenate	100 γ
DL-Tryptophan	100 mg.	Riboflavin	100 γ
DL-Alanine	100 mg.	Thiamine	100 γ
DL-Aspartic acid	50 mg.	Niacin	100 γ
Ammonium acetate	100 mg.	Pyridoxamine	200 γ
Fumaric acid	250 mg.	Biotin	0.2 γ
Sodium ethyl oxalacetate	250 mg.	*p*-Aminobenzoic acid	20 γ
Adenine	5 mg.	Folic acid	1 γ
Guanine	5 mg.	KCN	125 γ
Uracil	5 mg.	Water to	250 ml.

Inoculum was prepared by transferring from agar stab to a broth consisting of the Difco riboflavin assay medium supplemented with clarified skim milk (casein removed at pH 4.2). Cell growth obtained from 10 ml. of this broth was washed twice with sterile saline (centrifuge) and resuspended to give a reading of 70 on the Evelyn colorimeter using the No. 660 filter. One drop of this suspension was added per assay tube.

The assay was carried out using 5 ml. total volume, as this was found by Caswell *et al.*[20] to eliminate the frequent occurrence of maximum growth in tubes without vitamin B_{12} when the 10 ml. total volume is used. The assay tubes were autoclaved 8 minutes, incubated 40 hours at 37°, and titrated with 0.05 *N* NaOH.

The major advantage of this *L. lactis* Dorner tube method is the elimination of the necessity for preparing clarified tomato juice and enzymatic

[32] M. C. Nymon and W. A. Gortner, *J. Biol. Chem.* **163**, 277 (1946).

digests of casein. The composition of these preparations may vary from batch to batch. This method in its present form must be applied to a variety of natural materials before it can be completely evaluated.

b. Lactobacillus leichmannii 313 (7830)

Development. The most thoroughly tested method for vitamin B_{12} is the method adopted by the Pharmacopeia of the United States,[31] utilizing the organism *L. leichmannii* 313 (7830). This organism was observed to respond to thymidine[33, 34] whereas thymine was inactive. The details of this assay are given in the section entitled Standardization of Activity (p. 472).

Hoffmann *et al.*[35] demonstrated that for *L. leichmannii* 313 thymidine and crystalline vitamin B_{12} were interchangeable and that this organism may be useful as a sensitive test organism for vitamin B_{12}. Thymidine and vitamin B_{12} were also shown to be interchangeable for the growth of *L. lactis* Dorner (8000).[24, 25] A report by Kitay *et al.*[36] indicated that *L. leichmannii* 313 responded to the desoxyribosides of adenine, hypoxanthine, and cytosine as well as to intact desoxyribonucleic acid and thymidine.

Hoffmann *et al.*[37] first published a detailed method using *L. leichmannii* 313 (7830) for the assay of vitamin B_{12}. The medium used, in addition to the usual vitamins, minerals, carbohydrate, and buffers present in media for lactic acid bacteria, contained thioglycollic acid, Tween 80, and enzymatic digest of casein. The addition of thioglycollic acid to the medium promoted much heavier growth at submaximal levels of liver extract or vitamin B_{12}. Cysteine and ascorbic acid had similar effects but were somewhat inferior to thioglycollic acid. Thus these three reducing agents caused a several-fold increase in growth response to both vitamin B_{12} and liver extract. This was interpreted as indicating that reducing agents protected vitamin B_{12}, pure or contained in liver extract, from destruction during autoclaving. Newer information published by Broquist *et al.*[38] suggests that the protective effect may be of importance only for vitamin B_{12b} and that the effect on vitamin B_{12} is rather an activation or change of vitamin B_{12} to a form more potent for the organism. The effect of reducing agents on microbial growth promotion of vitamin B_{12} and vitamin B_{12b} will be discussed in Section X.

[33] E. E. Snell, E. Kitay, and W. S. McNutt, *J. Biol. Chem.* **175**, 473 (1948).
[34] L. D. Wright, H. R. Skeggs, and J. W. Huff, *J. Biol. Chem.* **175**, 475 (1948).
[35] C. E. Hoffmann, E. L. R. Stokstad, A. L. Franklin, and T. H. Jukes, *J. Biol. Chem.* **176**, 1465 (1948).
[36] E. Kitay, W. S. McNutt, and E. E. Snell, *J. Biol. Chem.* **177**, 993 (1949).
[37] C. E. Hoffmann, E. L. R. Stokstad, B. L. Hutchings, A. C. Dornbush, and T. H. Jukes, *J. Biol. Chem.* **181**, 635 (1951).
[38] H. P. Broquist, E. L. R. Stokstad, and T. H. Jukes, *Proc. Soc. Exptl. Biol. Med.* **76**, 806 (1951).

Hoffmann *et al.*[37] found that for routine assays with *L. leichmannii* 313 it was advisable to replace the enzymatic digest of casein with a butanol-extracted asparagus juice concentrate. This resulted in less growth in the blank tubes containing no added vitamin B_{12}. These investigators also describe a method for correcting for the growth due to desoxyribosides occurring in liver extracts together with vitamin B_{12}. Vitamin B_{12} was destroyed by heating with 0.2 *N* NaOH at 100° for 30 minutes under which conditions the desoxyribosides of thymine, guanine, and hypoxanthine are not affected. Liver extracts were assayed before and after this treatment, and the difference was ascribed to vitamin B_{12}. Assay of several liver extracts revealed that only about 3 % of the growth-promoting activity of liver extracts was due to desoxyribosides. *L. leichmannii* (4797) was also tested on this medium devised for *L. leichmannii* 313 (7830) and was found to require 48 hours to reach maximum growth as compared to 20 hours for *L. leichmannii* 313 (7830).

The U.S.P. microbiological assay method for vitamin B_{12} has continued to be the subject of study. Campbell *et al.*[38a] suggested a change in the experimental design of the method. These workers selected a portion of the titrimetric response curve which, when plotted on log-log paper, gave a straight line. Only three dilutions of the standard and three dilutions or less of the samples were used. Using such modifications as the randomization of tubes, the omission of tomato juice, and the range method of calculation, Campbell *et al.* were able to obtain limits of error as small as ±5 %. Frost *et al.*[38b] compared a rat-growth assay for vitamin B_{12} with the U.S.P. microbiological method. Studying the same U.S.P. liver extracts which were used in extensive collaborative tests of the U.S.P. microbiological method for vitamin B_{12}, these investigators found good correlation between the rat-growth method and the U.S.P. microbiological method. They further reported that vitamins B_{12} and B_{12b} gave equal and full response by both methods and that alkali destroyed over 90 % of the vitamin B_{12} as measured microbiologically or by the rat assay. The investigations of the University of Reading group on vitamin B_{12}-like compounds active for microorganisms but not for animals are of great importance with regard to valid microbiological assays for vitamin B_{12}. It must be recognized that all the organisms now in use for the assay of vitamin B_{12} respond to great or lesser extent to factors A, B, and C and pseudovitamin B_{12}. This includes bacteria of the lactic acid group, *E. coli* and *Euglena gracilis*. It is therefore of great importance to consider carefully all new organisms offered for the assay of vitamin B_{12}. Hamilton *et al.*[38c] discussed briefly the possibility of using

[38a] J. A. Campbell, J. M. McLaughlan, G. A. Clark, and C. W. Dunnett, *J. Am. Pharm. Assoc. Sci. Ed.* **42**, 276, 1953.

[38b] D. V. Frost, H. H. Fricke, and H. C. Spruth, *J. Nutrition* **49**, 107, 1953.

[38c] L. D. Hamilton, S. H. Hutner, and L. Provasoli, *Analyst* **77**, 618, 1952.

certain strains of chrysomonads (photosynthetic phagotrophic flagellated protozoa). These investigators expressed the hope that since chrysomonads were able to ingest large particles they would respond to all naturally bound forms of vitamin B_{12} in a manner similar to that of rats or chicks The chrysomonads are able to utilize intact proteins. Baile[38d] described experiments with the chrysomonad *Poteriochromonas stipitata*, showing that in a variety of samples of natural materials the chrysomonad assay gave consistently the lowest value for vitamin B_{12}. An ambitious and practical study of the use of the chrysomonad *Ochromonas malhamensis* was undertaken by Ford.[38e] When this procedure was compared with the *Euglena gracilis* and *E. coli* assays, it was found that factors A, B, C, and pseudovitamin B_{12} possessed considerable activity for all organisms except the ochromonad. The hope that the chrysomonads would be able to determine natural or bound vitamin B_{12} unavailable to other microorganisms as expressed by Hamilton *et al.* was not realized, since Ford found that vitamin B_{12} in sows' milk must first be liberated by a preliminary digestion of the milk with papain. Comparing the vitamin B_{12} content of natural materials as determined with *Ochromonas* and *E. coli*, Ford found that the two methods gave similar values for cows' milk, beef liver extract, and fish solubles; however, feces or urine gave significantly higher values by the *E. coli* method. It appears that the chrysomonad *Ochromonas malhamensis* assay for vitamin B_{12} offers for the first time a method specific for the cobalamins. The existence in nature of substances related to vitamin B_{12} which are inactive in animal nutrition but are active for the growth of certain microorganisms presents obvious problems in the interpretation of microbiological assays. These substances may be essentially absent from certain materials, such as liver extract, in which case *L. leichmannii* or *E. coli* may be used for the assay. However, if these substances are substantially present, as in the case of intestinal contents, the *Ochromonas* assay should be used, unless the interfering substances are first removed by paper electrophoresis. The main disadvantages of the *Ochromonas* method are the incubation time of 4 days and the general lack of familiarity of most workers with this type of organism.

c. Lactobacillus leichmannii (4797)

Skeggs *et al.*,[39] finding the use of the *L. lactis* Dorner (8000) assay procedure "difficult and unpredictable," investigated *L. leichmannii* (4797) as

[38d] D. Baile, mimeograph distributed by the National Dairy Research Laboratories, Long Island, 1953.

[38e] J. E. Ford, *Brit. J. Nutrition* **7**, 299 (1953).

[39] H. R. Skeggs, J. W. Huff, L. D. Wright, and D. K. Bosshardt, *J. Biol. Chem.* **176**, 1459 (1948).

a test organism for vitamin B_{12}. The medium was not completely chemically defined, as it contained an enzymatic digest as well as an acid hydrolyzate of casein. Growth was measured turbidimetrically after 24 hours. Reducing agents were not added to the medium. Ascorbic acid and air permitted growth of the organism in the absence of vitamin B_{12}, but the effects could be minimized by using an autoclaving period of 15 minutes in the assay procedure. Thymidine was interchangeable with vitamin B_{12}. Further investigations on the use of *L. leichmannii* (4797) for vitamin B_{12} assay were reported by Peeler *et al.*[40] These workers substituted crystalline amino

TABLE XI

Composition of Double-Strength Medium

Acid-hydrolyzed casein[a]	1.0 g.	Pyridoxal	400 γ
Glucose	4.0 g.	Riboflavin	200 γ
Tryptophan	20 mg.	Niacin	200 γ
Cystine	20 mg.	Pantothenic acid	200 γ
Adenine	1 mg.	Thiamine	200 γ
Guanine	1 mg.	Pteroylglutamic acid	100 γ
Xanthine	1 mg.	*p*-Aminobenzoic acid	100 γ
Uracil	1 mg.	Tween 80	0.2 ml.
Salts A	1 ml.	Thiomalic acid (crystallized)	100 mg.
Salts B	1 ml.	Guanylic acid	25 mg.
Sodium acetate (anhydrous)	1.2 g.	Distilled water to	100 ml.
Biotin	1 γ	pH 6.6 to 6.8	
Pyridoxine	400 γ		

[a] The preparation of the acid hydrolyzed casein requires special care, since repeated carbon treatment of the hydrolyzates results in inadequate growth of the organism. One hundred grams of Labco vitamin-free casein are refluxed for 8 to 10 hours with 1000 ml. of 6 *N* HCl. The HCl is removed under reduced pressure, the volume is restored with water, and the evaporation under vacuum is repeated. The hydrolyzate is diluted to 800 ml., adjusted to pH 3.0, and treated after filtration with 10 g. of Darco G60 for 30 minutes.

acids for the casein hydrolyzates used by Skeggs *et al.*[39] On the amino acid basal medium the maximum growth obtainable with excess vitamin B_{12} corresponded to a galvanometer reading of about 50% light transmission. After the addition of charcoal-treated tomato juice to furnish unidentified factors, a growth corresponding to 13% light transmission was obtained with adequate vitamin B_{12}. Thioglycollic and ascorbic acids did not duplicate the effect of the charcoal-treated tomato juice. Considerably more growth was obtained at all levels of vitamin B_{12} by increasing the amount of $FeSO_4 \cdot 7H_2O$ to 0.4 mg. per milliliter and the cysteine to 1 mg. per milliliter, amounts quite in excess of the usual. The best initial pH of the basal medium for best response to vitamin B_{12} was reported to be pH 5.5. Although

[40] H. T. Peeler, H. Yacowitz, and L. C. Norris, *Proc. Soc. Exptl. Biol. Med.* **72**, 515 (1949).

no mention was made of the possible function of the high levels of ferrous sulfate and cysteine other than that they were essential to keep the medium in the "necessary reduced state," it appears quite likely that these compounds directly affect vitamin B_{12}.

The assay method as described by Peeler *et al.*[40] is about one-tenth as sensitive as the *L. leichmannii* 313 (7830) method.[31] The authors claim that the method gives quite consistent and reproducible results. Fourteen assays on a vitamin B_{12} concentrate gave values ranging from 7.69 to 8.54 γ per milliliter, whereas ten assay values on a U.S.P. liver extract ranged from 8.18 to 9.44 γ per milliliter. This method has not been applied to a large number of samples or widely used by other workers.

TABLE XII

SENSITIVITY OF VARIOUS ASSAY METHODS FOR VITAMIN B_{12}

Method	Amount of vitamin B_{12} required per milliliter of sample, mγ	Interfering substances, maximum permissible amount per milliliters of diluted sample extract, γ	
E. coli pad method[28]	5.0	Methionine	500
E. coli tube method[43, 44]	0.5	Methionine	10
L. leichmannii (4797)[41]	0.1	Desoxyribosides[a]	0.5
		Intact DNA	40
L. leichmannii 313 (7830)[31]	0.01	Desoxyribosides	0.5
Euglena gracilis[45, 46]	0.01	None known	
L. lactis Dorner (8000)[20]	0.02	Desoxyribosides	0.5

[a] Desoxyribosides of thymine, adenine, hypoxanthine, and cytosine.[36]

Skeggs[41] has recently reported several improvements in the vitamin B_{12} assay medium used with *L. leichmannii* (4797). The medium finally developed is shown in Table XI. It was found that the enzymatic digest of casein could be omitted from the medium. Guanylic acid as well as uridylic acid and ribonucleic acid markedly improve the vitamin B_{12} response curve. Several reducing agents, among which were thioglycollic acid, cysteine, ascorbic acid, ethylenediaminetetraacetic acid, and thiomalic acid, were equally effective in preventing the destruction[42] of vitamin B_{12}. Thiomalic

[41] H. R. Skeggs, H. M. Nepple, K. A. Valentik, J. W. Huff, and L. D. Wright, *J. J. Biol. Chem.* **184**, 211 (1950).

[42] This may be partially an activation as well as a prevention of destruction, as will be discussed in Section X.

[43] B. D. Davis and E. S. Mingioli, *J. Bacteriol.* **60**, 17 (1950).

[44] P. R. Burkholder, *Science* **114**, 459 (1951).

[45] S. H. Hutner, L. Provasoli, E. L. R. Stokstad, C. E. Hoffmann, M. Belt, A. L. Franklin, and T. H. Jukes, *Proc. Soc. Exptl. Biol. Med.* **70**, 118 (1949).

[46] W. J. Robbins A. Hervey, and M. E. Stebbins, *Bull. Torrey Botan. Club* **77**, 423 (1950).

acid was chosen as a reducing agent, since it also gave a growth response similar to non-reducing dicarboxylic acids. By direct comparison it was shown to be superior to thioglycollic acid for this organism. The method of inoculation finally adopted is worthy of special attention. The organism is carried in stock culture in skim milk supplemented with 1 % Difco tryptose. For assay purposes the organism is transferred daily in this milk medium. The inoculum is prepared by suspending 0.1 ml. of a 24-hour milk culture in 10 ml. of sterile saline from which a second dilution of 1 to 10 is made. One drop of this second dilution is added to each assay tube. This has given good reproduceability with consistently less blank trouble than the U.S.P. method,[31] however, it is less sensitive (see Table XII).

d. Euglena gracilis

Hutner *et al.*[45] have shown that *Euglena gracilis* var. *bacillaris* exhibited a quantitative response to vitamin B_{12}. The unique advantage of the method

TABLE XIII

Basal Medium for *Euglena gracilis*

	Per 1 l. final medium (pH 6.5)
$NH_4H_2PO_4$	0.8 g.
Potassium citrate monohydrate	0.2 g.
$MgSO_4 \cdot 7H_2O$	0.2 g.
Sodium butyrate	2.0 g.
Monosodium glutamate	1.0 g.
$CaCl_2$	0.1 g.
$FeSO_4 \cdot 7H_2O$	20 mg.
$MnSO_4 \cdot H_2O$	6 mg.
$CoSO_4 \cdot 7H_2O$	5 mg.
$ZnCl_2$	0.8 mg.
$Na_2MoO_4 \cdot 2H_2O$	1.0 mg.
$CuSO_4 \cdot 5H_2O$	0.08 mg.
Thiamine chloride	0.1 mg.

is that, for *E. gracilis*, thymidine up to 10 γ per milliliter does not support growth. This is in contrast to the lactobacilli which grow in the absence of vitamin B_{12} on levels of desoxyribosides as low as 0.2 γ per milliliter of medium. The *Euglena gracilis* method utilizes a relatively simple medium, as shown in Table XIII. The organism is grown in small 100 × 13-mm. test tubes which must be exposed continually to light. To provide the required illumination the racks of tubes were placed on glass plates over large fluorescent lights. The temperature ranged from 25° to 31°. Temperatures of 32° or over caused inhibitory effects.

The standard response curve starts at 0.0015 γ of vitamin B_{12} per milliliter and goes up to 0.5 γ per milliliter.

The *Euglena gracilis* method has been studied further by Robbins *et al.*[46] Casein hydrolyzate or DL-alanine and a supplement of the B vitamins were added to the basal medium of Hutner *et al.*[45] Better growth response at the higher levels of vitamin B_{12} was apparent when the assays were heated at 100° instead of autoclaving. These authors, as well as using the conventional tube method, developed a method utilizing a medium solidified with agar.

Filter paper disks dipped in vitamin B_{12} test solutions were placed on the solid medium seeded with *Euglena gracilis*, and the areas of growth around the disk were measured. This disk method was rather insensitive, requiring extracts containing at least 2 mγ of vitamin B_{12} per milliliter. An incubation period of 7 days at 25° to 28° was used by Robbins *et al.*[46] for both disk and tube methods.

In a recent paper, Robbins *et al.*[46] report the application of the *Euglena gracilis* method to a variety of natural products. Satisfactory assays were obtained on natural materials, in contrast to the experience of Picken and Bauriedel,[47] who found the *E. gracilis* tube method applicable only to relatively pure materials. Robbins *et al.*[48] found oysters and clams excellent sources of vitamin B_{12}. Pure cultures of blue-green algae produce vitamin B_{12} and were suggested to be the primary source of vitamin B_{12} for aquatic animals. Clams contained vitamin B_{12} in a bound form from which the vitamin can be released by treatment with 0.1 N HCl.

The *Euglena gracilis* method requires an incubation time of at least 4 days,[45] and sometimes as long as 7 days.[46] This is a major disadvantage when a large number of assays are required. On the other hand, from the report of Hutner and the chromatographic studies of Picken and Bauriedel,[47] it appears that the *Euglena gracilis* method is the most specific method for vitamin B_{12}.

e. Escherichia coli

Davis and Mingioli[43] isolated by means of the penicillin method[49] several ultraviolet-induced mutants of *E. coli* which require vitamin B_{12}. One mutant in particular, No. 113-3, showed a rapid and reproducible response and was suggested as a possible assay organism. The vitamin B_{12}-requiring mutants of *E. coli* require about 0.5 γ of vitamin B_{12} per milliliter of medium and hence are slightly less sensitive than *L. leichmannii* (4797) and other lactobacilli (Table XII). *E. coli* 113-3 differs from the lactobacilli in that it does not respond to thymidine or alkaline hydrolyzates of desoxynucleic

[47] J. C. Picken and W. R. Bauriedel, *Proc. Soc. Exptl. Biol. Med.* **75**, 511 (1950).

[48] W. J. Robbins, A. Hervey, and M. E. Stebbins, *Bull. Torrey Botan. Club* **78**, 363 (1951).

acid.[49] Methionine, however, does interfere in the assay, 20 γ of methionine per milliliter of medium giving full growth. Burkholder[44] has tested the *E. coli* mutant 113-3 for vitamin B_{12} assay, using a medium containing, in addition to the salts and carbohydrate recommended by Davis and Mingioli,[43] five amino acids and thioglycollate. He recommends that the assay tubes be shaken during the incubation. The organism responded "in a similar fashion" to vitamins B_{12} and B_{12a}. Assays of blood serum proteins, animal tissue extracts, and various preparations of bacteria and algae yielded satisfactory results in comparison to duplicated assays by *Euglena gracilis*.

Shive has referred to a sulfanilamide inhibition assay for vitamin B_{12}, using wild type *E. coli*.[50] Vitamin B_{12} and methionine are interchangeable in reversing the inhibition of sulfanilamide. The details of this method have not been published, although the method has been used extensively by the Texas workers.

2. Cup-Plate Methods for Assay of Vitamin B_{12}

Cup-plate methods in general have certain advantages over tube methods. Perhaps the chief advantage is the adaptability of the cup-plate technique to the routine assay of large numbers of samples. In the assay of vitamin B_{12} by tube methods using lactobacilli, the degree of aeration, the accumulation of carbon dioxide and peroxides, and the oxidation-reduction potential have given assay troubles. In the cup-plate procedure or any procedure using solid media these factors are readily controllable. The first complete cup-plate method for vitamin B_{12} was described by Foster *et al.*[51] These workers used a stabilized culture of *L. lactis* Dorner, A.T.C.C. No. 10,697, and a chemically defined medium using crystalline amino acids. The inclusion of 2% sodium chloride in the growth medium was found to eliminate the response of the organism to thymidine and desoxynucleic acid. One-half per cent ascorbic acid also failed to cause interference in the test. The authors state that impure materials containing various LLD-active substances do not necessarily give equal values by the cup-plate and tube methods, using the same organism.

Cuthbertson and Lloyd[52] have studied a variety of factors influencing the response of *L. lactis* to vitamin B_{12} in the cup-plate procedure. The thickness of the solid medium, i.e., the oxygen concentration, the concentration of interfering desoxyribosides which must be less than 5 γ per milli-

[49] B. D. Davis, *Proc. Natl. Acad. Sci. U. S.* **35**, 1 (1949).

[50] W. Shive, Presented at N. Y. Academy of Sciences, Symposium on Antimetabolites, Feb. 11, 1949.

[51] J. C. Foster, J. A. Lally, and H. B. Woodruff, *Science* **110**, 507 (1949).

[52] W. F. J. Cuthbertson and J. T. Lloyd, *J. Gen. Microbiol.* **5**, 416 (1951).

liter of sample extract, and treatment of the inoculum must be carefully controlled. A detailed procedure for the cup-plate assay with *L. lactis* (8000) has been recently published by Cuthbertson, Pegler, and Lloyd.[53] The basal medium is not chemically defined in that it contains tomato juice. Ascorbic acid is added as a reducing agent. Only a limited series of assays have been reported with this method. It does not appear to be applicable to *Streptomyces* fermentation liquors which contain vitamin B_{12c}.[54] In the plate test vitamin B_{12c} was three to four times as active as vitamin B_{12}, although clinically its activity is approximately the same.[55] These authors did not attempt to eliminate interference due to desoxyribosides, since such interference can be readily detected visually. Desoxyribosides gave faint diffuse zones, and solutions containing both vitamin B_{12} and desoxyribosides gave a dense zone of growth in the center surrounded by a diffuse area due to desoxyribosides. At very high concentrations of desoxyribosides the center vitamin B_{12} growth zone is obscured.

Bessell *et al.*[56] have briefly described the application of the Davis *E. coli* mutant to the assay of vitamin B_{12}, using the cup-plate technique. These authors report that the method is relatively free of the usual assay troubles encountered with lactobacilli, and the reproducibility is comparable. The simple medium, inorganic salts and glucose, was a distinct advantage. Methionine at a concentration of 10 to 100 mg. per milliliter gave a growth zone. More recently Harrison *et al.*[57] reported in detail on the *E. coli* cup-plate method, pointing out the superiority of the method for determination of the naturally occurring vitamin B_{12c} (nitrosocobalamin). Vitamin B_{12c} which occurs in *Streptomyces* liquors and liver extracts gave assay values four to five times as high by the *L. lactis* Dorner plate method as those obtained by tube methods. With a *L. leichmannii* 313 plate method vitamin B_{12c} gave fuzzy, poorly defined zones. With the *E. coli* cup-plate assay, however, vitamin B_{12c} is approximately equivalent on a weight basis to vitamin B_{12}. The simple salts-glucose medium used earlier for the *E. coli* cup-plate assay[56] was fortified with a variety of trace minerals and with asparagine which increased the density of the growth zones. The method appears to be remarkably free of effects of non-specific interfering substances and inexplicable variations in growth at various levels of the same sample commonly observed in lactobacilli methods. The method is somewhat less sensitive than the *E. coli* pad-plate method (Table XII) requiring 10 mγ per milliliter of sample extract. Methionine at a concentration of 1 mg. per milliliter of extract interferes with the assay.

[53] W. F. J. Cuthbertson, H. F. Pegler, and J. T. Lloyd, *Analyst* **76**, 133 (1951).
[54] E. L. Smith, *Proc. Roy. Soc. Med.* **43**, 535 (1950).
[55] C. C. Ungley, D. L. Mollin, and J. V. Dacie, *Lancet* **I**, 353 (1950).
[56] C. J. Bessell, E. Harrison, and K. A. Lees, *Chemistry & Industry* **1950**, 561.
[57] E. Harrison, K. A. Lees, and F. Wood, *Analyst* **76**, 696 (1951).

3. *Escherichia coli* Pad-Plate Method

As indicated in a preliminary report,[28] a method has been developed in our laboratories which has proved of particular value for the rapid assay of large numbers of samples. The method utilizes the vitamin B_{12}-requiring mutant, *E. coli* 113-3,[43] and a simple mineral salts-glucose medium solidified with agar. The innovation which eliminates much of the time-consuming mechanical manipulation usually involved in microbiological assay methods is the use of paper pads originally developed for antibiotic assay[58] with plates containing the solid assay medium. This technique possesses all the general advantages of plate methods mentioned earlier and in addition eliminates the boring of holes in the agar, removal of the plugs, and sealing of the holes with hot agar. Besides the advances in technique the method

TABLE XIV

Basal Medium For Pad-Plate Assay with *E. coli*

K_2HPO_4	7.0 g.
KH_2PO_4	3.0 g.
Na_3-citrate·$3H_2O$	0.5 g.
$MgSO_4 \cdot 7H_2O$	0.1 g.
$(NH_4)_2SO_4$	1.0 g.
Glucose	2.0 g.
Difco agar	15 g.
Water	1000 ml.
Adjust to pH 7.0	

has the fundamental advantage that vitamin B_{12}, vitamin B_{12b}, vitamin B_{12c}, amminocobalamin, and sulfite-treated cyanocobalamin all have equal activity.

The basal medium is given in Table XIV. The glucose and the salts with the agar and most of the water are autoclaved separately for 5 to 10 minutes, combined, and inoculated at 45° to 48° with two 3-mm. loops of *E. coli* slant culture per liter of medium. The inoculum slants which are also used for stock cultures consist of Difco peptone 0.6 g., BBL trypticase 0.4 g., Difco yeast extract 0.3 g., beef extract 0.15 g., glucose 0.1 g., and agar 1.5 g. per 100 ml.

Standard solutions or extracts of test samples are pipetted on paper pads, 0.01 ml. or 0.02 ml. on the 6.5-mm. size, and 0.05 or 0.1 ml. on the 13-mm. size (No. 740-E, available from Carl Schleicher and Schuell, New York).

The following amounts of pure vitamin B_{12} give zone diameters which form a straight line plotted against the log of the dose: 0.5, 1.0, 2.5, 5.0, 10, and 20 mγ. Over this range doubling the concentration gave an increase

[58] J. G. Vincent and H. W. Vincent, *Proc. Soc. Exptl. Biol. Med.* **55**, 162 (1944).

of 1.8 to 2 mm. in zone diameter. The zones are measured with pointed calipers with an accuracy of ±0.1 mm. With the larger pads, standard and sample solutions can be five times as dilute as with the smaller pads, since a larger volume of liquid can be pipetted on the large pads. The liquid added to the pad is allowed to dry before the pad is placed on the surface of the agar. This permits the use of solvents other than water. The basal medium immediately after inoculation is poured into 35 × 16-cm. flat plates. The plates which have stainless steel edges cemented to plate glass need only to be thoroughly cleaned, not sterilized. Large Pyrex plates may

TABLE XV

Comparison of Vitamin B_{12} Assays by the *E. coli* Pad Method and *L. leichmannii* (4797) Tube Method

Sample	Pad method, γ/ml. or g.	Tube method (Skeggs *et al.*[41]), γ/ml. or g.
Commercial liver extracts		
1	21.8	19.8
2	24.4	22.4
3	25	18.2
4	17.3	16.2
5	12.9	13.2
6	9.3	9.8
7	28.3	22.0
8	26.8	23.4
9	27.6	26.0
10	31.0	21.0
11	23.7	23.4
Antibiotic feed supplement A	1.7	1.1
Antibiotic feed supplement B	1.5	0.7
Fermentation solids	25.7	14.6

also be used, but usually these give variations in depth of medium which affect zone size. About eighteen samples at two levels in duplicate can be assayed on one plate together with a standard curve in duplicate.

The plates are incubated at 37°, and zones can be measured at any time from 5 hours to 4 days of incubation.

Test solutions containing, after dilution, 1 mg. per milliliter of methionine, or more, cannot be wholly satisfactorily assayed. The methionine interference can be readily detected by the diffuse zone of growth which surrounds the vitamin B_{12} zone, but at this high concentration of methionine the vitamin-B_{12}-zone size is affected. Table XV shows several comparative assays done by the pad method and the *L. leichmannii* (4797) method.[41] The higher values for the fermentation samples reflect the preponderance of vitamin B_{12b} in these samples. In the *L. leichmannii* (4797)

assay pure vitamin B_{12b} autoclaved in the medium is only 50 % to 70 % as active as vitamin B_{12} whereas with the pad method the two forms have equal activity.

After approximately a year's use of the method it appears to have the following major advantages:

1. Other than the flat plates no special glassware or equipment such as colorimeters is needed.

2. Because of ease and simplicity large numbers of assays can be obtained with a minimum of time and effort.

3. Pads containing the standard amounts of vitamin B_{12} can be prepared in advance. The standard curve covers a forty-fold range of concentration.

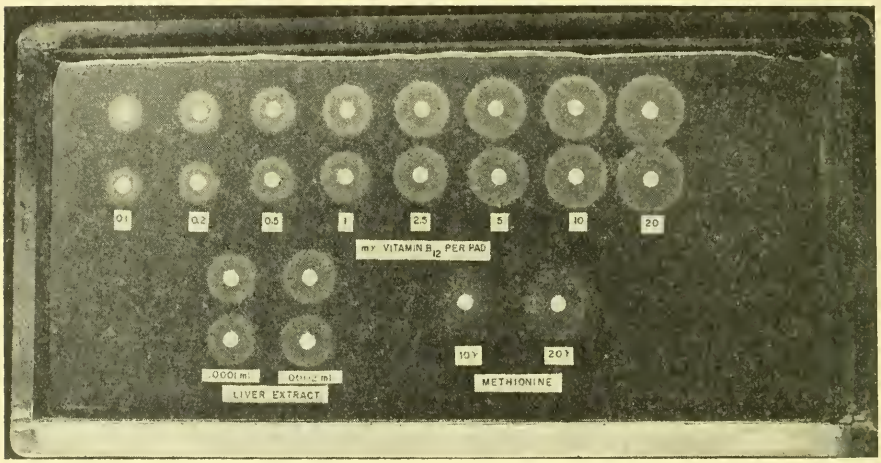


FIG. 6. Vitamin B_{12} determination by the pad-plate method. The liver extracts were diluted 1 to 100 before pipetting 0.01 and 0.02 ml. on the pads. Note diffuse edges of the readily recognized methionine zones.

4. Methionine is the only substance known to give a non-specific growth response, and this interference when it occurs is visually apparent.

5. Pure or crude forms of the vitamin need not be heated prior to assay, in fact, aseptic conditions in general are not essential.

6. Of greatest fundamental value is the equality of response of the organism to the various forms of the vitamin.

Figure 6 shows an assay plate containing standard curves, two liver extracts, and methionine. The diameters of the zones due to pure vitamin B_{12} in Fig. 6 are plotted against the logarithm of the dose and a straight line obtained as shown in Fig. 7.

4. SAMPLE TREATMENT

A suitable extraction procedure to release the various forms of vitamin B_{12} from natural materials has not been generally agreed upon. Various

enzyme preparations, almost all of them proteolytic, have been proposed[40, 59-61] for the release of the vitamin activity. Peeler *et al.*[40] found a pancreatin treatment of animal products and a papain-takadiastase treatment of vegetable materials to be superior to hot- or cold-water extractions at various pH's. Scheid and Schweigert[60] found considerable difficulty in obtaining consistent results after enzyme digestion. Pancreatin gave approximately a twofold increase in the vitamin B_{12} potency of beef liver and increases in other meat products. A recent report by these workers[62] is perhaps the most thorough report on release with various enzyme prepara-

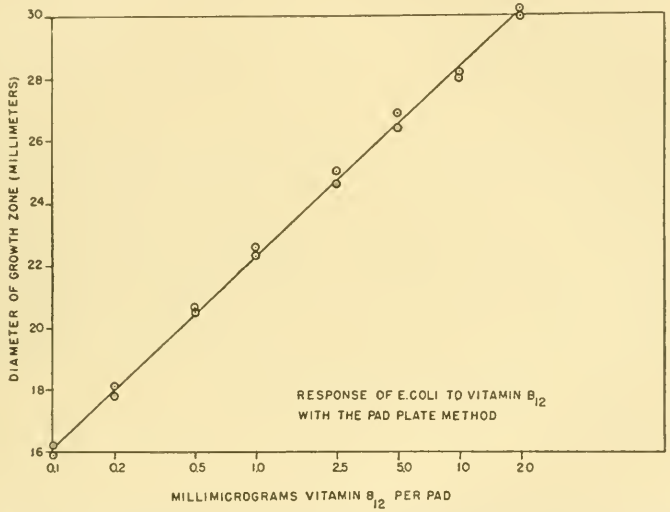


FIG. 7. The response of *E. coli* 113-3 to vitamin B_{12} in the pad-plate method. Measurements were taken from plate shown in Fig. 2.

tions and heat treatments. Crude preparations of trypsin, pancreatin, chick pancreas, takadiastase, and filtrates of proteolytic bacteria all increased the apparent vitamin B_{12} content of beef liver, beef round, and a few other meats approximately twofold. Autoclaving the tissues at neutral pH in phosphate buffer resulted in values only 5 to 10% less than the enzyme various treatments. In an extensive collaborative study carried out by the Association of Official Agricultural Chemists,[63] hot aqueous extraction was found superior to trypsin digestion. The most consistent and highest values were obtained by autoclaving 0.1 to 1.0 g. of sample 15 minutes with 25

[59] H. T. Thompson, L. S. Dietrich, and C. A. Elvehjem, *J. Biol. Chem.* **184**, 175 (1950).
[60] H. E. Scheid and B. S. Schweigert, *J. Biol. Chem.* **185**, 1 (1950).
[61] J. R. Couch and O. Olcese, *J. Nutrition* **42**, 337 (1950).
[62] H. E. Scheid and B. S. Schweigert, *J. Biol. Chem.* **193**, 299 (1951).
[63] C. H. Krieger, Personal communication.

ml. of 0.1 *M* phosphate-citrate buffer which contained 0.1 % sodium bisulfite (*meta*). This was also superior to autoclaving for the same length of time with distilled water. Harrison *et al.*[57] used with the *E. coli* cup-plate method a steaming period of 30 minutes at pH 5 to 6 in the presence of potassium cyanide. The presence of a small amount of cyanide has been reported by Wijmenga *et al.*[64] to facilitate the release of vitamin B_{12} forms from natural materials.

Hot-water extraction in all reports releases about 80 % or more of the vitamin B_{12} activity. It has not been determined whether the added increment resulting from the proteolytic action of crude enzyme preparations is due to vitamin B_{12} itself or to desoxyribosides. It is quite possible that the "battery" of enzymes in the various crude preparations would release many of the active soluble fragments of desoxynucleic acid or intact desoxynucleic acid which is also active. Furthermore, vitamin B_{12} activity has been shown to be synthesized by contaminating microorganisms in digests even in the presence of toluene,[65] and enzyme preparations contribute a significant amount of vitamin B_{12}.[62] It appears that release with enzymes which require 1 to 2 days should not be used until further study indicates a definite superiority. On the basis of present data autoclaving for 5 minutes or steaming for 30 minutes with phosphate, acetate, or citrate buffer between pH 5 and 6 will give satisfactory release of vitamin B_{12}. The use of cyanide or sulfite should be further investigated.

VIII. Standardization of Activity

THOMAS H. JUKES and WILLIAM L. WILLIAMS

The official U.S.P. qualitative and quantitative procedure for estimation of pure crystalline vitamin B_{12} is as follows:[1]

Vitamin B_{12} is a cobalt-containing substance usually produced by the growth of suitable microbial organisms, or obtained from liver. When assayed by the method described below, it has a purity of not less than 95 %, calculated on the anhydrous basis.

Description. Vitamin B_{12} occurs as dark-red crystals or as a crystalline powder. The anhydrous compound is very hygroscopic and when exposed to air it may absorb about 12 % of water.

[64] H. G. Wijmenga, W. L. C. Veer, and J. Lens, *Biochim. et Biophys. Acta* **6**, 229 (1950).

[65] D. V. Tappan, U. J. Lewis, and C. A. Elvehjem, *Abstr. Papers 119th Meeting, Am. Chem. Soc.*, p. 20A, 1951.

[1] U. S. Pharmacopeia, 14th revision, p. 660, 1950.

Solubility. One gram of vitamin B_{12} dissolves in about 80 ml. of water. It is soluble in alcohol, but is insoluble in acetone, in chloroform, and in ether.

Identification. A. Determine the absorbency of the solution prepared for the assay in a 1-cm. quartz cell with a suitable spectrophotometer, using water as the blank. Maxima within ± 1 mμ are found at 278 and 361 mμ and within ± 4 mμ at 548 mμ. The ratios of the absorbencies so observed are as follows:

$$\frac{A_{361}}{A_{278}} \text{ is not less than 1.62 and not more than 1.88}$$

$$\frac{A_{361}}{A_{548}} \text{ is not less than 2.83 and not more than 3.45}$$

B. Fuse about 1 mg. of vitamin B_{12} with about 50 mg. of potassium bisulfate in a porcelain crucible. Cool, break up the mass with a glass rod, add 3 ml. of water, and dissolve by boiling. Add 1 drop of phenolphthalein T.S. and 10 % sodium hydroxide solution, dropwise, until just pink. Add 500 mg. of sodium acetate, 0.5 ml. of diluted acetic acid, and 0.5 ml. of nitroso-R-salt solution (1 in 500): a red or orange-red color appears at once. Add 0.5 ml. of hydrochloric acid, and boil for 1 minute; the red color persists.

Loss on drying. Weigh accurately, on a microbalance, about 5 mg. of vitamin B_{12} in a suitable microdrying vessel. Heat in a suitable vacuum-drying apparatus at 105° and at a pressure of not more than 5 mm. for 2 hours, cool, and weigh: it loses not more than 12 % of its weight.

Assay. Weigh accurately, on a microbalance, about 2 mg. of vitamin B_{12}, transfer to a 50-ml. volumetric flask with the aid of 15 or 20 ml. of water, add water to make exactly 50 ml., and mix well. Determine the absorbency of the solution in a 1 cm. quartz cell, at 361 mμ, with a suitable spectrophotometer, using water as the blank.

Calculate the per cent purity of vitamin B_{12} by the following formula:

$$\frac{A_{361}}{0.0207} \times \frac{1}{\text{wt. of sample (mg. per 10 ml.)}} \times \frac{100}{\text{(100-loss on drying)}}$$

L. leichmannii 313 (7830) *Tube Assay.* As mentioned earlier, a method utilizing *L. leichmannii* 313 (7830) has been adopted by the U.S.P.[2] and has been the subject of a collaborative study among several laboratories. A special culturing procedure for the test organism renders the assay particularly sensitive. The culture medium consists of 0.75 g. of yeast extract, 0.75 g. of peptone, 1 g. of dextrose, 0.2 g. of potassium biphosphate, 10 ml. of filtered tomato juice, and 100 mg. of Tween 80 in 100 ml. of water.

[2] U. S. Pharmacopeia, 14th revision, 3rd Supplement, p. 15, 1951.

The solution is adjusted to pH 6.8. The solid stab medium for stock cultures is obtained by simply adding 1.5 % agar to the culture medium.

From the fresh culture of *L. leichmannii* 313 (7830) obtained, for example, from the American Type Culture Collection, to inoculum for the assay tubes, the following procedure is observed:

1. At least ten successive transfers over a 2-week period are made in the stab medium incubating 16 to 24 hours at a constant temperature between 30° and 37°.

2. During use of the organism for routine assays it is transferred at least

TABLE XVI

COMPOSITION OF THE BASAL MEDIUM FOR THE U.S.P. VITAMIN B_{12} METHOD

Dextrose	10	g.	Riboflavin	250	γ
Sodium acetate	5	g.	Thiamine	250	γ
Acid-hydrolyzed casein	2.5	g.	Biotin	2.0	γ
Ascorbic acid	1	g.	Niacin	500	γ
Tween 80	0.5	g.	*p*-Aminobenzoic acid	500	γ
L-Cystine	100	mg.	Calcium pantothenate	250	γ
DL-Tryptophan	100	mg.	Pyridoxine·HCl	1000	γ
L-Asparagine	50	mg.	Pyridoxal·HCl	1000	γ
Adenine, guanine, uracil, each	5	mg.	Pyridoxamine·2HCl	200	γ
Xanthine	5	mg.	Tomato juice preparation[a]	50	ml.
Salt solution A	5	ml.	Water to	250	ml.
Salt solution B	5	ml.	Adjust to pH 6.0		

[a] Tomato juice preparation—3 l. of canned tomato juice is centrifuged and then filtered by suspending 10 to 20 g. of filter-aid in the solution and also using a filter-aid layer on a Büchner funnel. A clear straw-colored solution must be obtained. The solution is treated at pH 3.5 with 50 g. of activated charcoal and filtered, using filter-aid as previously described.

three times a week, at no time using a stab culture which is more than 4 days old.

3. Transfer from the stab culture 10 ml. of the described liquid culture medium and incubate 16 to 24 hours at a constant temperature between 30° and 37°.

Daily transfer of the stab culture has been found to be advisable. To prepare inoculum the cells are centrifuged from 10 ml. of culture medium and are washed twice with 10-ml. portions of previously sterilized single-strength basal medium, finally suspending in 10 ml. of the basal medium. The published procedure[2] fails to mention that the suspension should be diluted 1 to 10 in the basal medium at this point. Dilution of 1 to 20 frequently has been advisable. One drop of this suspension is added per assay tube.

The double-strength basal medium contains the ingredients shown in

Table XVI. This medium is added in 5-ml. portions to 20 × 150-mm. Pyrex test tubes and made to 10-ml. volume with test solutions and water. The assay tubes are autoclaved for 5 minutes at 121° to 123°. The first tube in the standard curve contains 0.01 mγ of vitamin B_{12}, the successive tubes containing increasing amounts of vitamin B_{12} by increments of 0.01 mγ and the last tube containing 0.1 mγ.

The organism requires 0.01 mγ of vitamin B_{12} per milliliter for maximum growth, whereas *Euglena gracilis* reaches approximately half-maximum growth at this concentration.[3] In experiments reported by Robbins *et al.*[4, 5] *Euglena gracilis* required 0.1 mγ per milliliter for maximum growth. Thus this present method using *L. leichmanni* 313 is at least as sensitive as the *Euglena gracilis* method. It is about ten times as sensitive as methods utilizing other lactobacilli (Table XII).

The sensitivity of the *L. leichmannii* 313 assay is a disadvantage under some conditions. In our laboratories, growth in tubes to which no vitamin B_{12} has been added is a frequent occurrence. The same blank troubles have been frequently encountered in other laboratories according to verbal reports. It is possible that reduction in the volume of medium used in the assay tube would help eliminate some of this erratic growth. Caswell *et al.*[6] found that, by reducing the volume of medium from 10 ml. to 5 ml., maximal growth in tubes containing no added vitamin B_{12} was completely eliminated and greater reproducibility of assay results with *L. lactis* Dorner (8000) was obtained. The U.S.P. method[2] recommends titrating the acid produced at 72 hours as a means of measuring growth. Undoubtedly turbidity measurements would prove satisfactory in a considerably shorter period of incubation. The American Association of Official Agricultural Chemists[7] has conducted a study of the U.S.P. method[2] using *L. leichmannii* 313 except that turbidity measurements of growth are used. Means should be investigated for the elimination of the clarified tomato juice, since the composition of this preparation has been found to vary with different batches. Tomato juice was eliminated from *L. lactis* Dorner (8000) media by a combination of fumaric acid, sodium ethyl oxalacetate, and DL-alanine.[6] With the improvements of a shorter assay period by using turbidimetric measurement of growth and elimination of the tomato juice prepa-

[3] S. H. Hutner, W. Provasoli, C. E. Hoffmann, M. Belt, A. L. Franklin, and T. H. Jukes, *Proc. Soc. Exptl. Biol. Med.* **70**, 118 (1949).

[4] W. J. Robbins, A. Hervey, and M. E. Stebbins, *Bull. Torrey Botan. Club* **77**, 423 (1950).

[5] W. J. Robbins, A. Hervey, and M. E. Stebbins, *Bull. Torrey Botan. Club* **78**, 363 (1951).

[6] M. C. Caswell, L. K. Koditschek, and D. Hendlin, *J. Biol. Chem.* **180**, 125 (1949).

[7] C. H. Krieger, Personal communication.

rations the *L. leichmannii* 313 assay method would indeed be a most sensitive and practical method for vitamin B_{12} determination.

IX. Occurrence in Food

THOMAS H. JUKES and WILLIAM L. WILLIAMS

Early studies on the distribution of the anti-pernicious anemia factor (APF) in food are difficult or impossible to interpret because a response in pernicious anemia may be obtained to either folic acid or vitamin B_{12}. In fact, folic acid is more readily utilized than vitamin B_{12} by the oral route in this disease. Therefore, one wonders whether the responses first obtained by Minot and Murphy to the ingestion of large quantities of beef liver may have been due to the folic acid content of this food rather than to the presence of vitamin B_{12}.[1] Anti-anemic effects were reported by various investigators for extracts prepared from kidney,[2] brain, salivary glands, saliva,[3] pancreas, and spinach.[4]

The "extrinsic factor," which now appears to be vitamin B_{12}, was measured by its effect in producing a response in pernicious anemia when fed together with gastric juice. However, folic acid present in such foods could also produce a response whether or not gastric juice was added, and it seems reasonable to conclude that the so-called extrinsic factor activity of yeast must have been due in large part to its content of folic acid. Other sources of the extrinsic factor include lean meat, milk, and crude casein, and the activity of these sources was probably due to their vitamin B_{12} content, since they are low in folic acid.

The distribution of APF as measured by the growth response of chicks on an all-vegetable diet led to the compilation of information which was later shown to be related to the presence of vitamin B_{12}. In this manner fish meal, fish solubles, meat scrap, milk, liver, cow manure, the dried rumen contents obtained from cattle, and fermented chicken manure were shown to be sources of vitamin B_{12}. Alaska herring meal appeared to be a better source than certain other types of fish meal.

Studies by Cary and Hartman[5] with factor X led to the following conclusions as a result of assays with young rats on a basal diet deficient in vitamin B_{12}:

[1] G. R. Minot and W. P. Murphy, *J. Am. Med. Assoc.* **87**, 470 (1926).
[2] W. S. McCann, *Proc. Soc. Exptl. Biol. Med.* **25**, 255 (1928).
[3] W. Hofer and L. Hofer, *Klin. Wochschr.* **13**, 6101 (1934).
[4] S. Okadas, M. Shamato, and F. Yanasef, *Nagoya J. Med. Sci.* **11**, 147 (1937).
[5] C. A. Cary and A. M. Hartman, *Yearbook Agr. 1943–1947*, *U. S. Dept. Agr.* p. 783.

Foods and feed that do not contain the still unidentified factor	Foods and feed that do contain the unidentified factor
White flour	Milk
Enriched white flour	Skim milk (liquid or dried)
Whole-wheat flour	Cheese (cottage, Swiss, Cheddar)
Yeast (baker's or brewer's)	Liver extracts
Wheat bran	Beef muscle
Corn meal (yellow)	Pork muscle
Soybean oil meal	Egg yolk
Linseed oil meal	Lettuce
Egg white (heat-coagulated)	Alfalfa and alfalfa hays
Carrots	Timothy hays
Tomatoes	Kentucky bluegrass

The presence of the factor in the green leafy materials listed above is not in accordance with results reported by other investigators who used biological assays with rats, chicks, or bacteria.

Zucker and Zucker[6] made an extensive study of the distribution of "zoopherin," now presumed to be identical with vitamin B_{12}, by measuring the growth of young rats weaned from mothers on an all-vegetable diet. Hog liver and calf liver were found to be very good sources, and good amounts of zoopherin were present in other meat products including beef round, calf spleen, and calf thymus. Various marine animals including starfish, oysters, the eggs of *Artemia salina*, winkles, and sand worms also contain substantial amounts of zoopherin.

Following the isolation of vitamin B_{12} and the development of the *L. leichmannii*[7, 8] and *Euglena gracilis*,[9] microbiological assay procedures, exploration of the distribution of the vitamin in foods was greatly accelerated. Some information is present in Table XVII. It is not known to what extent the microbiological assay values were complicated by the presence of pseudo-vitamin B_{12}.[10] However, it is evident that most green leafy materials contain no vitamin B_{12} detectable by the sensitive *Euglena gracilis* assay.

From the broad standpoint vitamin B_{12} may be regarded as a substance which is produced by microorganisms and not by the higher plants. Its existence in animal tissues occurs as a result of the ingestion, directly or indirectly, of products of microbial fermentation. The presence of vitamin

[6] T. F. Zucker and L. M. Zucker, *Vitamins and Hormones* **8**, 1 (1950).

[7] C. E. Hoffmann, E. L. R. Stokstad, A. L. Franklin, and T. H. Jukes, *J. Biol. Chem.* **176**, 1465 (1948).

[8] H. R. Skeggs, H. M. Nepple, K. A. Valentik, J. W. Huff, and L. D. Wright, *J. Biol. Chem.* **184**, 211 (1950).

[9] S. Hutner, W. Provasoli, E. L. R. Stokstad, C. E. Hoffmann, M. Belt, A. L. Franklin, and T. H. Jukes, *Proc. Soc. Exptl. Biol. Med.* **70**, 118 (1949).

[10] J. J. Pfiffner, D. G. Calkins, R. C. Peterson, O. D. Bird, V. McGlohon, and R. W. Stipek, *Abstr. Papers, 120th Meeting, Am. Chem. Soc.*, p. 22C (1951).

TABLE XVII

Vitamin B_{12} Content of Natural Materials

Type of assay	Material	Vitamin B_{12}		
		Micrograms per gram fresh material		
Euglena assay[11, 13]	Oysters	0.20 –0.46		
	Clams, untreated	0.028 –0.071		
	Clams, after heating with 0.1 *N* HCl	0.138 –0.180		
	Earthworms	0.08 –0.18		
	Shrimp	0.0055–0.0090		
		Root	Stem	Leaf
	Tomato	0.015	0.0002	0 in 0.26 g.
	Cabbage	0.01	0.0005	0 in 0.2 g.
	Celery	0.002	...	0 in 0.6 g.
	Pepper, green	0.002	0 in 0.4 g.	0 in 0.4 g.
	Kale	0.01	0.0002	0 in 0.2 g.
	Kohlrabi	0.01	0 in 0.6 g.	Less than 0.0001 in 4 g.
	Broccoli	0.01	0 in 0.5 g.	0 in 0.56 g.
	Leek	0.01	0 in 0.6 g.	0 in 0.6 g.
		Micrograms per kilogram or liter of fresh material		
	Soil	2 –15		
	Bone marrow, beef, yellow	2		
	Cow's milk	1 – 2		
	Clam juice	1.4–10		
	Roots of various garden vegetables	4 –10		
	Excised tomato roots grown in Pfeffer's solution	None		
	Pond water	0.1– 2		
		Micrograms per gram dry weight		
	Blue-green algae (*Plectonema nostocorum*)	0.06–0.07		
	Blue-green algae (*Calothrix parietina*)	0.64		
	Blue-green algae (*Aphanizomenon flos-aquae*)	0.285		
	Blue-green algae (*Diplocystis aeruginosa*)	0.24		
		Micrograms per gram solids		
Rat assay[6]	Hog liver	1.8		
	Calf liver	2.4		
	Liver residue from hot water extraction	0.3		
	Beef round	0.18		
	Starfish	0.18		
	Oysters	2.8		
	Artemia salina eggs	7.2		
	Winkles	1.2		
	Sandworms	5		
	Earthworms	1.1		

TABLE XVII—*Continued*

Type of assay	Material	Vitamin B_{12}	
		Micrograms per gram solids	
L. leichmannii assay[14]	Ling cod liver	0.43–0.88	
	Ling cod muscle	0.18	
	Halibut liver	0.52	
	Dogfish liver	0.1	
	Sockeye salmon liver	1.7	
	Sockeye salmon kidney	18.0	
	Sockeye salmon pyloric ceca	3.1	
	Sockeye salmon spleen	7.2	
	Sockeye salmon milt	0.48	
	Sockeye salmon eggs	1.7	
	Sockeye salmon stomach	1.7	
	Red cod muscle	0.12	
	Condensed fish solubles	0.02–0.67	
	Fish meals	0.17–1.5	
	Meat meal	0.17	
	Littleneck clams	2.5	
		Micrograms per 100 g. dry weight	
		(A)	(B)
Ray assay (A); *L. leichmannii* assay (B)[15]	Beef round (cooked$_4$	5.5	5.0, 7.9
	Beef tongue (cooked)	5.5	7.6
	Pork shoulder (cooked)	0.9	0.7, 3.0
	Pork ham (cooked)	2.2	2.9, 3.0
	Veal	3.6	3.0
	Horsemeat	7.5	7.0
	Fish solubles	40	15, 25
	Beef liver	50	47, 50
	Tomato juice	0	0
	Beef kidney		50
	Mutton (fresh)		8.8
	Beef heart		25
	Hog spleen		0, 9, 22
	Hog adrenal		15
		Micrograms per 100 g. dry weight	
L. leichmanii assay[15]	Crude casein	3, 7	
	Cow's milk	2	
	Cheddar cheese	2.5	
	Egg yolk	3	
	Sheep rumen contents[a]	100	
	Guinea pig feces[a]	230	
	Chicken manure[a]	450	
	Goat manure[a]	20	
	Cow manure[a]	47	
	Chicken breast (fresh)	5.3	
	Chicken leg (fresh)	5.2	
	Salmon	8.5	
	Oysters	15	
Euglena assay[16]	Beef liver	260	

[a] Possibly high due to presence of pseudovitamin B_{12}.[16]

TABLE XVII—*Concluded*

FOODS AS SOURCES OF VITAMIN B_{12}

Excellent Sources (0.5 γ or more per gram of dry matter)
- Mammalian liver and kidney
- Oysters
- Clams

Good Sources (0.05 to 0.5 γ per gram of dry matter)
- Lean beef
- Milk
- Veal
- Lamb
- Poultry meat
- Salt-water fish

Poor Sources (in many cases giving no response in the assay)
- Cereal grains
- Green leaves
- Vegetables
- Leguminous seeds
- Yeast

B_{12} in the blue-green algae is of great interest.[11] The existence of substantial quantities of the vitamin in this primitive form of life may indicate the source from which marine animals such as clams, oysters, other mollusks, and bony fish accumulate such comparatively large amounts of vitamin B_{12}. In terrestrial forms of life, the presence of the vitamin in soil microorganisms, in earthworms and even in soil itself indicates the sources from which vitamin B_{12} may reach the tissues of vertebrates. The manner in which ruminating animals obtain their supply of vitamin B_{12} is indicated by the role of cobalt in their nutrition. Evidently fermentation in the rumen, in which ingested soil bacteria may well play a part, serves to furnish these animals with the vitamin.

Robbins has speculated upon the production of vitamin B_{12} in pond mud by fermentation and its relation to the growth of vitamin B_{12}-requiring algal flagellates, typified by *Euglena gracilis*, in stagnant water.[11] The possible involvement of the vitamin in the growth of marine microorganisms is indicated by Sweeney's[12] observation that *Gymnodinium*, a marine dinoflagellate associated with "red tides," needs for growth an unidentified organic substance present in soil (? vitamin B_{12}), thus suggesting that "red tides" may be due to the occurrence of vitamin B_{12} in sea water at levels which favor the growth of *Gymnodinium*.

[11] W. J. Robbins, A. Hervey, and M. E. Stebbins, *Bull. Torrey Botan. Club* **78**, 363 (1951).
[12] B. M. Sweeney, *Am. J. Botany* **38**, 669 (1951).
[13] W. J. Robbins, A. Hervey, and M. E. Stebbin, *Bull. Torrey Botan. Club* **77**, 423 (1950).
[14] H. L. A. Tarr, B. A. Southcott, and P. W. Ney, *Food Technol.* **4**, 354 (1950).
[15] C. A. Elvehjem, *Proc. 2nd Conf. on Research, Am. Meat Inst., Univ. Chicago* p. 1 (1950).
[16] B. S. Schweigert, Personal communication.

X. Effects of Deficiency

THOMAS H. JUKES and WILLIAM L. WILLIAMS

A. METABOLIC ASPECTS AND FUNCTION OF VITAMIN B_{12} IN MICROORGANISMS

1. Purine and Pyrimidine Desoxyribosides

Thymidine was shown by several groups of investigators to be interchangeable with vitamin B_{12} for the growth of *L. lactis* Dorner[1, 2] and for other lactobacilli.[3, 4] The parent pyrimidine, thymine, under the same conditions of growth was inactive. These observations on microorganisms served as a basis for speculation on the mechanism of action of vitamin B_{12} in pernicious anemia. Thus, Wright *et al.*[2] suggested that vitamin B_{12} may function as a coenzyme in carrying out reactions leading to the conversion of thymine to thymidine. However, thymidine appears to be no more active than thymine in producing a response in pernicious anemia. Kitay *et al.*[4] showed that thymidine was not the only desoxyriboside which would replace vitamin B_{12} but that the desoxyribosides of adenine, hypoxanthine, and cytosine were effective for *L. leichmannii* 313, *L. acidophilus* 832, and *L. acidophilus* 4913. The existence of organisms such as *L. delbrueckii* 730[4] and *L. acidophilus* 204,[4, 5] which require thymidine but cannot grow with vitamin B_{12}, and the fact that thymidine does not specifically replace vitamin B_{12} cast considerable doubt on the theory of Wright *et al.*[2] mentioned above. The interchangeability of the desoxyribosides[5] and also the activity of the phosphorylated products, desoxyribotides,[6] suggest that these compounds may simply be providing the groups which they have in common, D-2-desoxyribose; however, tests of both the synthetic compound and a crude natural preparation indicate that D-2-ribose does not promote growth of organisms requiring the desoxyribosides.[5] No direct experimental evidence is available, indicating that vitamin B_{12} functions as a coenzyme for the biosynthesis of desoxyribosides.

2. Effect of Reducing Agents

Several reports have appeared indicating that chemical reducing agents and reducing conditions obtained by physical means will permit growth of various lactobacilli in the absence of vitamin B_{12}.[1, 4, 7] Koditschek *et al.*[7]

[1] W. Shive, J. M. Ravel, and R. E. Eakin, *J. Am. Chem. Soc.* **70**, 2614 (1948).
[2] L. D. Wright, H. R. Skeggs, and J. W. Huff, *J. Biol. Chem.* **175**, 475 (1948).
[3] E. E. Snell, E. Kitay, and W. S. McNutt, *J. Biol. Chem.* **175**, 473 (1948).
[4] E. Kitay, W. S. McNutt, and E. E. Snell, *J. Biol. Chem.* **177**, 993 (1949).
[5] E. Kitay, W. S. McNutt, and E. E. Snell, *J. Bacteriol.* **59**, 727 (1950).
[6] W. Shive, M. E. Sibley, and L. L. Rogers, *J. Am. Chem. Soc.* **73**, 867 (1951).
[7] L. K. Koditschek, D. Hendlin, and H. B. Woodruff, *J. Biol. Chem.* **179**, 1093 (1949).

found that *L. lactis* 8000 was affected in this manner by reducing agents and that the requirement for vitamin B_{12} was increased by oxidizing conditions or oxidizing agents. Kitay *et al.*[5] made a detailed study of ascorbic acid with several microorganisms. Ascorbic acid, thymidine, and vitamin B_{12} all promoted growth of three out of ten lactobacilli. For several others, for which thymidine and vitamin B_{12} gave similar effects, ascorbic acid was ineffective. For one organism, *L. acidophilus* 204, ascorbic acid permitted growth which was heavier and more rapid than that obtained with thymidine whereas vitamin B_{12} was inactive. *L. delbrueckii* 730 is an example of an organism for which neither vitamin B_{12} or ascorbic acid was effective in replacing thymidine.

Welch and Wilson[8] found that ascorbic acid replaced vitamin B_{12} for the growth of *L. leichmannii* only if the test medium contained an enzymatic casein digest. The potency of ascorbic acid was increased by autoclaving it with the medium. These observations served as the basis for the suggestion that ascorbic acid converted inactive oxidized products of vitamin B_{12} to microbially active forms. Other workers[5] found that for ten of fourteen cultures studied ascorbic acid must be autoclaved with the medium and that most organisms would not respond to ascorbic acid unless enzymatic casein digest was added. Four cultures of lactobacilli grew equally well or better when the ascorbic acid was added aseptically, and three strains would utilize ascorbic acid in the absence of enzymatic casein digest. These exceptions led Kitay *et al.*[5] to question the interpretation of Welch and Wilson[8] and to conclude that ascorbic acid could not be acting solely through the production of vitamin B_{12} from oxidized products in the enzymatic digest of casein since (a) it was active for several organisms, for example, *L. acidophilus* S., in the absence of such digests, and (b) it promoted growth of certain organisms typified by *L. acidophilus* 204 that could not utilize vitamin B_{12} in place of ascorbic acid. A partial explanation of the effects of ascorbic acid and other reducing agents may be that only below a certain oxidation-reduction potential can vitamin B_{12} be synthesized by organisms of the above type. This postulate must also include the assumption that the cells of organisms which utilize ascorbic acid, but not vitamin B_{12}, are impermeable to extracellular vitamin B_{12} and must rely on vitamin B_{12} synthesized within the cell in response to the presence of ascorbic acid. As has been pointed out[5] there is marked variation among microorganisms of the same genus in the quantitative requirement of vitamin B_{12} which most probably represents a difference in permeability to this high-molecular-weight vitamin.

The effect of reducing agents is closely connected with the relative microbiological activity of vitamins B_{12} and B_{12b} under various conditions. For

[8] A. D. Welch and M. F. Wilson, *Arch. Biochem.* **22**, 486 (1949).

L. leichmannii 4797,[9] *L. leichmannii* 313, and *L. acidophilus* 213,[5] vitamin B_{12b} was found to be less active than vitamin B_{12}. These results were obtained on a medium without added reducing agents. Hoffman *et al.*[10] had demonstrated considerably earlier that vitamin B_{12} and vitamin B_{12b} contained in liver extract were protected from destruction by thioglycollic acid. The apparent destruction took place when pure or crude samples were autoclaved in the medium without a reducing agent. The protective property of thioglycollic acid is most apparent in combination with basal medium[10] and is evidently of greater importance for vitamin B_{12b} than for vitamin B_{12}. In spite of earlier reports[9, 11] that vitamin B_{12} and vitamin B_{12b} were of approximately equal activity when added *aseptically* to microbiological assay medium, it appears well established at present that vitamin B_{12b} is considerably more potent under these conditions.[9, 12-14] In fact, for at least one organism, *L. leichmannii* 327 (A.T.C.C. 7831), vitamin B_{12b} is 20 % to 80 % more active than vitamin B_{12} under all conditions of assay and addition.[13] Loy and Kline[14] reported for *L. leichmannii* 313 that aseptically added vitamin B_{12b} was about 30 % more active than vitamin B_{12}, whereas the two forms were of equal activity when autoclaved in the medium containing ascorbic acid. A subsequent study by Broquist *et al.*[12] revealed certain points regarding the nature of the action of reducing agents such as thiomalic acid. Both vitamins B_{12} and B_{12b} had very low activity for *L. leichmannii* 4797 when autoclaved with medium without thiomalic acid. Thiomalic acid was added to samples of vitamin B_{12} and vitamin B_{12b} in a series of tubes which had been autoclaved once without thiomalic acid and the tubes were re-autoclaved. Vitamin B_{12} regained full microbiological activity, and vitamin B_{12b} regained a large part of its activity. This demonstrated that thiomalic acid was not protecting vitamin B_{12} but, when present in the medium during autoclaving, actually caused the formation of a microbiologically more potent form. By paper chromatography and microbiological activity this form was shown to be different from vitamin B_{12b}. Vitamin B_{12b} autoclaved in the medium with thiomalic acid was 60 % to 70 % as active as the product derived from vitamin B_{12} under these conditions. When both forms were added aseptically to previously heated medium containing thiomalic acid, vitamin B_{12b} was about five times more

[9] E. A. Kaczka, R. G. Denkewalter, A. Holland, and K. Folkers, *J. Am. Chem. Soc.* **73**, 335 (1951).

[10] C. E. Hoffmann, E. L. R. Stokstad, B. L. Hutchings, A. C. Dornbush, and T. H. Jukes, *J. Biol. Chem.* **181**, 635 (1951).

[11] D. Hendlin and M. H. Soars, *J. Biol. Chem.* **188**, 603 (1951).

[12] H. P. Broquist, E. L. R. Stokstad, and T. H. Jukes, *Proc. Soc. Exptl. Biol. Med.* **76**, 806 (1951).

[13] H. E. Scheid and B. S. Schweigert, *J. Biol. Chem.* **193**, 299 (1951).

[14] H. W. Loy, Jr., and O. L. Kline, *J. Assoc. Offic. Agr. Chemists* **34**, 225 (1951).

active than vitamin B_{12}. From this work[12] it thus appears that a highly microbiologically active form of vitamin B_{12} exists which under assay conditions is more stable than existing known forms.

3. Methionine

Davis and Mingioli,[15] studying mutants of *E. coli*, have demonstrated that vitamin B_{12} and methionine serve as alternate growth factors for certain mutants. These authors interpret this as meaning that vitamin B_{12} functions as a coenzyme in the synthesis or transfer of labile methyl groups, since all the vitamin B_{12}-requiring mutants are blocked between homocysteine and methionine. *E. coli* mutants which can utilize either homocysteine or methionine can synthesize their requirements for vitamin B_{12}, since their synthetic mechanism is at an earlier stage in the pathway of biosynthesis of homocysteine. The observation that two compounds are interchangeable for growth of a microorganism does not at all reveal which compound functions in the synthesis of the other. The existence of two *E. coli* mutants, Nos. 113-30 and 26-20, which grow very rapidly with methionine but considerably more slowly with vitamin B_{12}[15] strongly suggests that vitamin B_{12} is the biocatalyst and methionine the product. An impermeability to vitamin B_{12} was ruled out by the observation that the above mutants readily absorb vitamin B_{12} from the medium and hence a biochemical defect in vitamin B_{12} utilization exists. Davis and Mingioli[15] postulate that this defect is a limited ability to form a vitamin B_{12} coenzyme. Choline, betaine, and creatine do not produce growth with the methionine-vitamin B_{12} mutants and therefore do not serve in *E. coli* as methyl donors for the methylation of homocysteine. Desoxyribosides, in marked contrast to the situation with lactobacilli, do not substitute for vitamin B_{12} in the nutrition of *E. coli* mutants. Since desoxyribosides exist in the cells of both groups of organisms, and it seems quite unlikely that they would be synthesized by totally different mechanisms, this is indeed an interesting relationship. Davis and Mingioli[15] consider it improbable that methionine-vitamin B_{12} mutants, for example, *E. coli* 113-3, have only a partial block in the synthesis of vitamin B_{12} allowing enough for synthesis of desoxyribosides but not for that of methionine.

Cell suspensions of the mutant *E. coli* 113-3 have been studied by Oginsky *et al.*[16] regarding the oxidative capacities of the cells as influenced by vitamin B_{12}. After storage for 5 days or longer of cells grown under moderately anaerobic conditions, the rates of oxidation of acetate, pyruvate, oxalacetate, succinate, glutamate, malate, fumarate, acetoacetate and stearate

[15] B. D. Davis and E. S. Mingioli, *J. Bacteriol.* **60**, 17 (1950).

[16] E. L. Oginsky, P. H. Smith, N. E. Tonhazy, W. W. Umbreit, H. C. Lichstein, and S. F. Carson, *J. Bacteriol.* **61**, 581 (1951).

were increased by the addition of vitamin B_{12}. The final total amount of oxygen uptake was not influenced but only the *rate*. The amount of vitamin B_{12} involved in the process was extremely small; approximately only 2 molecules per bacterial cell.

4. Forms of Vitamin B_{12} with Greater Potency for Microorganisms Than for Animals

The first report of forms of vitamin B_{12} which are more active for microorganisms than for animals was that of Coates *et al.*[17] These workers found that both dried rumen contents and dried calf feces with *L. leichmannii* 4797[18] gave assay values approximately five times as high as the values obtained by chick growth, whereas values obtained with the *E. coli* assay[19] were about twenty times as high. The extracts assayed were treated with cyanide to convert all forms to the cyano derivatives. Using a concentrate prepared by adsorbing a water extract of calf feces on charcoal and eluting with hot 75% acetone, paper chromatography in the presence of cyanide showed the existence of two forms active for *E. coli* besides vitamin B_{12}.[20] These were called fractions A and B. Fraction A was later[21] shown to be about five times as potent for *Euglena gracilis* and *E. coli* as for *L. leichmannii* 4797. Amounts up to 200 γ per kilogram of diet were inactive for chicks. For chicks fraction A antagonized one-fifth its own weight of vitamin B_{12}.[22] Fraction B was inactive for chicks orally, and both fraction A and B were about one-tenth as active as vitamin B_{12} when injected intramuscularly in chicks. In one pernicious anemia patient, fraction A appeared to have only slightly less activity than vitamin B_{12}. Further chromatographic studies[21] revealed the presence of a fourth fraction, C, which was about fifteen times as active for *E. coli* as for *L. leichmannii* 4797 and with only slight activity for *Euglena gracilis*. It should be emphasized that fractions A, B, and C all contain cyanide and, therefore, differ from vitamin B_{12} in some other portion of the molecule.

Pfiffner *et al.*[23] have reported that an organism, as yet incompletely identified, isolated from bovine rumen contents produces six cobalt-contain-

[17] M. E. Coates, J. E. Ford, G. F. Harrison, S. K. Kon, J. W. G. Porter, W. F. J. Cuthbertson, and H. F. Pegler, *Biochem. J.* **49**, lxvii (1951).

[18] H. R. Skeggs, H. M. Nepple, K. A. Valentik, J. W. Huff, and L. D. Wright, *J. Biol. Chem.* **184**, 211 (1950).

[19] C. J. Bessell, E. Harrison, and K. A. Lees, *Chemistry & Industry* **1950**, 561.

[20] J. E. Ford, S. K. Kon, and J. W. G. Porter, *Biochem. J.* **50**, ix (1951).

[21] J. E. Ford and J. W. G. Porter, 304th Meeting, Biochemical Society, London, 1952; *Biochem. J.* (In press).

[22] M. E. Coates, J. E. Ford, G. F. Harrison, S. K. Kon, and J. W. G. Porter, 304th Meeting, Biochemical Society, London, 1952; *Biochem. J.* (In press).

[23] J. J. Pfiffner, D. G. Calkins, R. C. Peterson, D. D. Bird, V. McGlohon, and R. W. Stipek, *Abstr. Papers 120th Meeting, Am. Chem. Soc.* p. 22C (1951).

ing pigments belonging to the vitamin B_{12} group. Two of these pigments were obtained in crystalline form and corresponded to the cyano and hydroxo forms, thereby bearing the same relationship to each other as vitamin B_{12} does to vitamin B_{12b}. Although the microbiological potency of these forms for *L. leichmannii* and *L. lactis* Dorner was approximately that of vitamin B_{12}, the pigments were totally inactive for growth of chicks and in the rat antihemorrhagic kidney test. For some strains of *L. acidophilus* these compounds, designated pseudovitamin B_{12}, were more active than vitamin B_{12}. This definitely higher potency for these lactobacilli opposes the view that the pseudovitamin B_{12} compounds serve as precursors to animal-active forms of vitamin B_{12}. Dion *et al.*[24] found evidence from examination of products resulting from acid degradation studies that pseudovitamin B_{12} differed from vitamin B_{12} solely in the occurrence of adenine instead of 5,6-dimethylbenzimidazole in the nucleotide portion of the molecule.

A third group[25] reported that a new form of vitamin B_{12} was produced in rat feces by feeding 100 mg. of cobalt sulfate per kilogram of diet. This fecal form of vitamin B_{12} was inactive for rat growth but had activity for *L. leichmannii* and chicks similar to vitamin B_{12}.

In a later report, these workers named this fecal form of vitamin B_{12}, vitamin B_{12f}.[25a] A crude preparation of vitamin B_{12f} gave a spot on being chromatographed on paper which had the same R_f value as Pfiffner's pseudovitamin B_{12}. In contrast to their earlier report, they found that this crude preparation does not possess growth-promoting activities for chicks. A substance resembling vitamin B_{12f} chromatographically was found to be present in the feces of several farm animals and man.

The recent reports of the University of Reading group[25b, c, d] clarified much of the confusion regarding forms of vitamin B_{12} which differ from vitamin B_{12} in the cobalamin part of the molecule. This group prepared extremely pure samples of factors A, B, C, and pseudovitamin B_{12}. Following the published directions, they also prepared a vitamin B_{12f} concentrate. Using paper ionophoresis to purify and characterize the various substances, Holdsworth of the University of Reading group found that vitamin B_{12f} is a crude mixture of factor A and pseudovitamin B_{12}. Crystalline preparations of pseudovitamin B_{12} also contained factor A. Vitamin B_{12m} concen-

[24] H. W. Dion, D. G. Calkins, and J. J. Pfiffner, *J. Am. Chem. Soc.* **74**, 1108 (1952).
[25] U. J. Lewis, D. V. Tappan, and C. A. Elvehjem, *J. Biol. Chem.* **194**, 539 (1952).
[25a] U. J. Lewis, D. V. Tappan, and C. A. Elvehjem, *J. Biol. Chem.* **199**, 517 (1952).
[25b] E. S. Holdsworth, *Nature* **171**, 148 (1953).
[25c] J. E. Ford, E. S. Holdsworth, S. L. Kon, and J. W. G. Porter, *Nature* **171**, 150 (1953).
[25d] J. E. Ford, *Nature* **171**, 149 (1953).

trated from pig manure by Wymenga[25e] was found to be a mixture of pseudovitamin B_{12} and factor B with a small amount of vitamin B_{12} itself. Factor WR of Wymenga is also a crude mixture containing factor A, pseudovitamin B_{12}, and vitamin B_{12}. The significance of factor WR was its preparation from beef liver. Thus, the designations of vitamin B_{12m}, factor WR, and vitamin B_{12f} should be discarded in favor of the substances prepared in pure form and characterized as to their position after electrophoresis on paper under defined conditions. None of the forms of vitamin B_{12}, factor A, B, C, and pseudovitamin B_{12} possesses significant activity for animals. These forms most commonly occur in the species of many different animals and in several cases have been demonstrated to exist in urine. For example, the vitamin B_{12} activity of cow urine is made up entirely of substances other than vitamin B_{12}. Calf feces contain fraction A with some pseudovitamin B_{12} and a trace of vitamin B_{12}.

5. Antimetabolites of Vitamin B_{12}

Woolley[26] found that 1,2-dichloro-4,5-diaminobenzene was inhibitory to organisms which do not show a nutritional requirement for riboflavin or vitamin B_{12}. It failed to retard growth of organisms which require both these vitamins. Woolley[26] presents convincing evidence that dichlorodiaminobenzene is an antimetabolite of 1,2-dimethyl-4,5-diaminobenzene contained within the structure of the two vitamins and pictured as a metabolic precursor of them. Dimethyldiaminobenzene was found to have some activity in replacing vitamin B_{12} for *L. leichmannii* 313 and *E. gracilis*. Mice whose nutritional needs may be compared to the lactobacilli which require both riboflavin and vitamin B_{12} were likewise very resistant to dichlorodiaminobenzene. They tolerated a level of 0.5% in their ration in addition to daily injections of 6 mg. Table XVIII from the excellent book of Woolley[27] on antimetabolites shows the unique correlation of the toxicity of dichlorodiaminobenzene with nutritional requirements for riboflavin and vitamin B_{12}. Direct evidence that dimethyldiaminobenzene participates in the biosynthesis of vitamin B_{12} is the observation[28] that in growing *Bacillus megatherium* cultures additions of the compound increased synthesis of the vitamin without affecting growth. Further structural analogs of dimethyldiaminobenzene were synthesized by Woolley and Pringle,[29, 30] many for

[25e] H. G. Wymenga, Vitamin B_{12} and Other Factors, Thesis, University of Utrecht, 1951.

[26] D. W. Woolley, *J. Exptl. Med.* **93**, 13 (1951).

[27] D. W. Woolley, A Study of Antimetabolites. John Wiley and Sons, New York, 1952.

[28] D. W. Woolley, *Proc. Soc. Exptl. Biol. Med.* **75**, 745 (1950).

[29] D. W. Woolley and A. Pringle, *Federation Proc.* **10**, 272 (1951).

[30] D. W. Woolley and A. Pringle, *J. Biol. Chem.* **194**, 729 (1952).

the first time. The antimetabolite properties of thirty-five of these compounds were tested against bacteria, chiefly *Staphylococcus aureus*. Conversion of one amino group to alkylamino or to acylamino resulted in elimination of the toxic properties. The replacement of an amino group by nitro or hydroxyl gave active growth inhibitors. As the structure of the antimetabolite became further removed from dimethyldiaminobenzene the

TABLE XVIII

Organism	Amount of analog, γ/ml.	Riboflavin requirement	B_{12} requirement
Lactobacillus brevis	6	None	None
Lactobacillus arabinosus	10	None	None
Saccharomyces cereviseae	6	None	None
Staphylococcus aureus	6	None	None
Streptococcus faecalis R.	40	None	None
Bacillus tenuis	20	None	None
Chlorella vulgaris	10	None	None
Ophiostoma multiannulatum mutant 1671	20	None	None
Leuconostoc mesenteroides	4	None	None
Xanthomonas pelargonii	1	None	None
Corynebacterium fascians	1	None	None
Corynebacterium michiganense	0.6	None	None
Pseudomonas angulata	100	None	None
Pseudomonas tabaci	30	None	None
Agrobacterium tumefaciens	60	None	None
Escherichia coli	200	None	None
Proteus 4	100	None	None
Shigella sonnei	200	None	None
Salmonella typhimurium	100	None	None
Lactobacillus casei	140	Required	None
Hemolytic streptococcus H69D	180	Required	None
Euglena gracilis	100	None	Required
Lactobacillus leichmannii 313	No effect at 300[a]	Required	Required
Lactobacillus bifidis 4963	No effect at 300	Required	Required
Lactobacillus lactis Dorner (8000)	No effect at 300	Required	Required

[a] This was the largest concentration which could be dissolved in test media.

toxic properties became irreversible by dimethyldiaminobenzene. However, these compounds still showed a marked reduction in the synthesis of vitamin B_{12} in growing cultures of *B. megatherium* just as dichlorodiaminobenzene has been found to do.[28]

Beiler *et al.*[31] report that treatment of a dilute solution of vitamin B_{12} with hydrogen peroxide formed an antagonist of vitamin B_{12}. The inhibitory properties of the reaction mixture were qualitatively competitively

[31] J. M. Beiler, J. N. Moss, and G. J. Martin, *Science* **114**, 122 (1951).

reversed by vitamin B_{12}. No attempt was made to remove hydrogen peroxide from the reaction mixtures before testing on *L. leichamannii* 4797, and according to the description of the procedure significant quantities of hydrogen peroxide were evidently added to the growth tubes. This is particularly important, since Koditschek *et al.*[7] have shown that as little as 10 γ of hydrogen peroxide per milliliter would completely inhibit *L. lactis* 8000 and that smaller amounts gave readily measurable inhibition. This inhibition by hydrogen peroxide, moreover, was competitively reversed by vitamin B_{12} over a considerable range of concentration. Until demonstrated to the contrary it appears quite likely that the inhibition observed by Beiler *et al.*[31] was due to residual hydrogen peroxide.

Studying microorganisms which require vitamin B_{12}, Hendlin and Soars[32] have found that 1,2-dimethyl-4,5-diaminobenzene is inhibitory and that it could be reversed by vitamin B_{12}. The toxic properties of 5,6-dimethylbenzimidazole and its analogs were not reversed by vitamin B_{12}. From the observation of Emerson *et al.*[33] that dimethyldiaminobenzene possessed vitamin B_{12} activity for the rat when fed in the diet and from Woolley's demonstration[26, 27] on the precursor role of this compound, it would appear that the animal activity of this compound is due to conversion to vitamin B_{12} by the intestinal flora. As Hendlin and Soars point out,[32] an alternate possibility is that, since dimethyldiaminobenzene is toxic to vitamin B_{12}-requiring organisms, such organisms are inhibited in the intestinal tract, allowing more vitamin B_{12} to become available to the animal.

XI. Pathology

A. CHICKS

THOMAS H. JUKES and WILLIAM L. WILLIAMS

A number of specific deformities have been described in chick embryos developing in eggs laid by hens on deficient diets. A diminution in hatchability was noted as the soybean meal content of the diet of hens was raised from 0 to 40% of the diet.[1]

The effect was reversed when cow manure was added to the diet.[2] Further investigations[3] were made with the basal diet containing 30% soybean meal, and, of 104 embryos examined, 12 had dystrophic leg muscles without

[32] D. Hendlin and M. H. Soars, *J. Bacteriol.* **62**, 633 (1951).

[33] G. Emerson, N. G. Brink, F. W. Hally, F. Koniuszy, D. Heyl, and K. Folkers *J. Am. Chem. Soc.* **72**, 3084 (1950).

[1] D. Whitson, H. W. Titus, and H. R. Bird, *Poultry Sci.* **25**, 52 (1946).

[2] D. Whitson, H. W. Titus, and H. R. Bird, *Poultry Sci.* **25**, 143 (1946).

[3] H. R. Bird, M. Rubin, D. Whitson, and S. K. Haynes, *Poultry Sci.* **25**, 285 (1946).

"micromelia." A large number of the chicks which hatched showed kidney damage with deposition of urates in the ureters. Normal development of embryos and chicks was noted when the diet contained fish meal. The leg deformity termed perosis was seen in many of the newly hatched progeny of hens which had been on all-vegetable diets containing soybean meal for more than 16 weeks. A supplement of herring fish meal markedly reduced the incidence of the deformity.[4] Purified diets consisting principally of sucrose and soybean protein were used for hens by Olcese and coworkers[5] in a study of the effects of vitamin B_{12} concentrates on embryonic development. A high incidence of myoatrophy of the leg was seen in the deficient

FIG. 8. Embryos with myoatrophy (left and right) and normal embryo (center). Removed from eggs on eighteenth day of incubation. (From Olcese *et al.*[5])

embryos (Fig. 8), characterized by very slender legs and by hemorrhages in the muscles and the cartilage. Another sign of the deficiency included high mortality during the sixteenth to eighteenth days of incubation with the embryos dying in the malposition "head between thighs." The abnormalities were prevented by adding concentrates which supplied between 10 and 20 γ of vitamin B_{12} per kilogram of diet.

B. RATS

THOMAS H. JUKES and WILLIAM L. WILLIAMS

Investigations were made into the causes of the high mortality in vitamin B_{12}-deficient rats in the period immediately following weaning.[6] No

[4] J. S. Carver, J. McGinnis, and R. J. Evans, *Poultry Sci.* **26**, 91 (1947).

[5] O. Olcese, J. R. Couch, J. H. Quisenberry, and P. B. Pearson, *J. Nutrition* **41**, 423 (1950).

lesions that could account for death were found in the internal organs, and autopsies and histological studies did not reveal the cause of death. Hematological examinations showed various abnormalities, especially leucopenia and granulocytopenia, which were closely correlated with mortality, but there was no evidence of infection, and death could not be attributed directly to the leucopenia. Hemoglobin levels and red blood cell counts were also lowered. Injections of vitamin B_{12} usually brought about rapid improvement and restored the blood picture to normal. In some instances the injection of 0.5 mg. of folic acid twice weekly resulted in improvement. Perhaps folic acid had a sparing effect upon the residual amounts of vitamin B_{12} in the tissues.

C. HUMAN BEINGS

FRANK H. BETHELL

1. Introduction

The pathologic manifestations of vitamin B_{12} deficiency in man are so interrelated with those of pteroylglutamic acid deficiency that, for the most part, no clear separation of the two can be made. This is particularly true of hemopoietic disturbances resulting in megaloblastic anemia, which represent the most common and clinically the most important evidences of deficiency of either B_{12} or PGA. However, like PGA, B_{12} is concerned with metabolic processes involving tissues other than the hemopoietic system, and through such functions, distinctions may be made between certain manifestations of lack of B_{12} and those of PGA. Thus, there is considerable evidence that B_{12} is concerned with growth of children, and the role of B_{12} deficiency in producing the neurologic lesions of pernicious anemia seems to be well established. Whether other neuropathies characterized by sensory disturbances which have been benefited by large doses of B_{12} are attributable to a lack of the vitamin or to a defect in its metabolism is still an unsettled question.

2. Mechanisms

The usual mechanisms which may be concerned in the production of most vitamin deficient states are applicable to vitamin B_{12}. These may be summarized as: limited sources of supply available to the organism; interference with or impairment of absorption; destructive or inhibiting influences; metabolic derangements affecting its conservation, storage, or utilization; imbalance between the substance in question and other metabolites upon which its function depends; and situations giving rise to

[6] H. J. Borson, D. Singman, S. Lepkovsky, M. K. Dimick, V. Gase, and R. Perry, *Am. J. Physiol.* **162**, 714 (1950).

excessive requirement. Certain apparently unique and, for the most part, poorly understood metabolic phenomena characterize the relationship of the human organism to B_{12}. It is these phenomena which comprise the subject matter for a discussion of the pathologic physiology of B_{12} deficiency.

Macrocytic anemias associated with megaloblastosis of the bone marrow have been considered systematically in relation to deficiency of PGA. They will be referred to in this section only in connection with the role of lack of B_{12} in their pathogenesis. It is a widely held opinion, which is shared by the writer, that megaloblastosis and other marrow changes characteristic of pernicious anemia and related conditions are the direct result of what may be termed PGA metabolic dysfunction. This does not necessarily imply an actual deficiency of PGA, since the enzymatic function of this vitamin is dependent upon its presence in biologically active form, as well as upon the completion of biologic reactions which require the participation of other substances, such as B_{12}.

Dietary deficiency of B_{12} might be presumed to occur quite frequently, since sources of the vitamin are limited essentially to foods of animal origin. That clinical evidences of such deficiency are quite rare suggests strongly that at least a major portion of the human requirement of B_{12} may be supplied by intestinal bacterial synthesis. Nevertheless, patients are encountered with macrocytic anemia whose diets have been almost devoid of animal protein and who respond to the parenteral or oral administration of B_{12}. The effectiveness of parenteral therapy[7-9] might be explained by associated intestinal disturbances with poor absorption of the vitamin, but the clear-cut responses to orally administered doses, such as 5 γ daily,[10-12] indicate that impaired absorption need not be the major cause of nutritional B_{12} deficiency. It may be postulated that such patients either do not obtain the usual amount of B_{12} from bacterial sources, perhaps owing to alteration of intestinal flora, or that, because of concomitant nutritional deficiencies, their requirement of the vitamin may be abnormally large.

3. Pernicious Anemia

In pernicious anemia the metabolic defect is primarily concerned with failure to assimilate B_{12} present in the alimentary tract. The demonstration

[7] T. D. Spies, R. M. Suarez, G. Garcia Lopez, F. Milanes, R. E. Stone, R. Lopez Toca, T. Aramburu, and S. Kartus, *J. Am. Med. Assoc.* **139**, 521 (1949).

[8] E. Jones, W. J. Darby, and J. R. Totter, *Blood* **4**, 827 (1949).

[9] J. C. Patel, *Brit. Med. J.* **II**, 934 (1948).

[10] G. A. Goldsmith, *Proc. 3rd Intern. Congr. Haematol.* Cambridge, England, 1950, Grune & Stratton, New York, p. 11, 1951.

[11] S. Chaudhuri, *Brit. Med. J.* **II**, 825 (1951).

[12] F. H. Bethell, M. E. Swendseid, S. Miller, and A. A. Cintron-Rivera, *Ann. Internal Med.* **35**, 518 (1951).

that normal human gastric juice administered to pernicious anemia patients together with crystalline B_{12} is followed by a therapeutic response unobtainable when the vitamin is given without gastric juice[13, 14] supports the theory of Castle concerning the role of a gastric secretory product, the intrinsic factor, in the maintenance of normal hemopoiesis.[15] Although a considerable amount of information has been acquired concerning the physical properties of intrinsic factor, it has not been isolated in pure form and little is known of its chemical nature. Recently, its close association with gastric mucoprotein has been demonstrated by Glass and associates.[16]

The extrinsic factor of Castle has been shown by these clinical experiments with gastric juice, as well as by others, in which derivatives of animal duodenal and gastric mucosa were employed,[17, 18] to be vitamin B_{12} or one of its analogs. Furthermore, since B_{12} alone is effective when administered parenterally to pernicious anemia patients, the inference is clear that the erythrocyte-maturing factor of liver, postulated by Castle and associates as the product of interaction between intrinsic and extrinsic factors,[19] is also B_{12}, perhaps present in "bound" form, but not altered chemically by its association with intrinsic factor. If this view is correct, and its validity seems to have been well established by studies concerned with the isolation and characterization of B_{12}, as well as by clinical investigations, then it must be concluded that the role of intrinsic factor is to render B_{12} available for absorption, or to facilitate its absorption from the alimentary tract.

Progress has been made toward the elucidation of this problem by the demonstration of a binding action of gastric juice and of gastric and intestinal derivatives on B_{12}. The complex so formed fails to support the growth of organisms, such as mutant strains of *E. coli*, which require an exogenous source of the vitamin. The protective effect of intrinsic factor on B_{12} might be exerted in two ways. The vitamin might be spared from utilization by intestinal bacteria, or from inactivation by their products, and in either case become available to the host. Moreover, the presence of free B_{12} in the proximal portion of the intestine should enable organisms

[13] L. Berk, W. B. Castle, A. D. Welch, R. W. Heinle, R. Anker, and M. Epstein, *New Engl. Med. J.* **239**, 911 (1948).

[14] B. E. Hall, E. H. Morgan, and D. C. Campbell, *Proc. Staff Meetings Mayo Clinic* **24**, 99 (1949).

[15] W. B. Castle, *Science* **82**, 159 (1935).

[16] G. B. J. Glass, L. J. Boyd, M. A. Rubinstein, and C. S. Svigals, *Science* **115**, 101 (1952).

[17] F. H. Bethell, M. E. Swendseid, M. C. Meyers, R. B. Neligh, and H. G. Richards, *Univ. Hosp. Bull., Michigan* **15**, 49 (1949).

[18] B. E. Hall, F. H. Bethell, E. H. Morgan, D. C. Campbell, M. E. Swendseid, S. Miller, and A. A. Cintron-Rivera, *Proc. Staff Meetings Mayo Clinic* **25**, 105 (1950).

[19] W. B. Castle, W. C. Townsend, and C. W. Heath, *Am. J. Med. Sci.* **180**, 305 (1930).

which require B_{12} to establish themselves at such sites and conceivably produce absorbable substances which are injurious to the host.

The ability of gastric juice and derivatives of gastric tissue to form an unstable combination with B_{12} was first demonstrated by Ternberg and Eakin.[20] These authors postulated the existence of a specific substance responsible for the B_{12} binding phenomenon and applied to it the term *apoerythein*. They considered that *apoerythein* is the intrinsic factor, although their conclusions and hypotheses were unsupported by clinical evidence. It has since been shown by Beerstecher[21] that saliva also contains a substance capable of forming a complex with B_{12} which renders the vitamin inactive in supporting microbial growth. However, Beerstecher's inference that the intrinsic factor activity (*apoerythein*) of gastric juice is derived from saliva, which in pernicious anemia patients undergoes inactivation because of lack of hydrochloric acid in the gastric secretion, is completely unsupported by any evidence and must be considered untenable. Saliva has no intrinsic factor activity when tested in patients with pernicious anemia, and, moreover, gastric achlorhydria is of common occurrence in persons who do not have pernicious anemia.

Lysozyme, derived from egg white, was shown by C. E. Meyer and associates[22] to possess the property of combining with B_{12}, but the complex so formed is no more effective in the treatment of pernicious anemia than the equivalent amount of orally administered free vitamin.[23] The existence of any relationship between binding capacity and intrinsic factor activity, even in the case of stomach tissue derivatives, has been questioned by Prusoff and associates.[24] These authors, by chemical fractionation of desiccated defatted hog stomach, have obtained materials which possess high binding power but are said to have low therapeutic effect as sources of intrinsic factor. However, the reported observations in substantiation of their claim are scanty. It has been the experience of others[12] that, with the methods of concentration employed, a parallelism exists between the intrinsic factor activity of gastric and duodenal preparations and their capacity to combine with B_{12}.

Recently, significant observations on the B_{12} binding phenomenon have been reported by Chow and Davis.[25] These authors and their associates[26, 27]

[20] J. L. Ternberg and R. E. Eakin, *J. Am. Chem. Soc.* **71**, 3858 (1949).

[21] E. Beerstecher, Jr., and S. Altgelt, *J. Biol. Chem.* **189**, 31 (1951).

[22] C. E. Meyer, S. H. Eppstein, F. H. Bethell, and B. E. Hall, *Federation Proc.* **9**, 205 (1950).

[23] B. E. Hall, *Brit. Med. J.* **II**, 585 (1950).

[24] W. H. Prusoff, G. C. Meacham, R. W. Heinle, and A. D. Welch, *Abstr. 118th Meeting, Am. Chem. Soc. Chciago* p. 27A (1950).

[25] B. F. Chow and R. L. Davis, *Proc. Scient. Sessions 7th Ann. Meeting, Natl. Vitamin Found.* p. 17 (1952).

have succeeded in determining the fate of orally or parenterally administered B_{12} by employing cobalt 60-labeled B_{12} and measuring the radioactivity in tissues, urine, and feces. They have emphasized the importance of attempting to differentiate between true binding capacity from an inhibitory effect on the microbiological test which may be exerted by certain substances. Yeast nucleic acid, chondroitin sulfuric acid, and heparin, as well as gastric juice and lysozyme, were shown to combine with B_{12} so as to make the vitamin unavailable to the test organisms. They confirmed the observation of Bird and Hoevet[28] that the combination of lysozyme and B_{12} when subjected to dialysis underwent ready dissociation and almost all the B_{12} was recoverable in the dialyzate. On the other hand, the complex formed by gastric juice and B_{12} was stable under the experimental conditions and yielded very little dialyzable B_{12}. The recovery percentages of B_{12} in the dialyzates when yeast nucleic acid, chondroitin sulfuric acid, and heparin were employed as binding agents were intermediate between the high value obtained with lysozyme and the minimum recovered in the case of gastric juice. Chow and Davis, by measuring urinary and fecal radioactivity, were also able to show that, in rats, absorption of cobalt 60-labeled B_{12} was greater when the vitamin was administered by mouth in combination with yeast nucleic acid than when it was given in the free form. These observations are of great interest, but the authors' conclusion that the techniques employed in their studies ". . . may result eventually in the discovery of compounds with the therapeutic properties of Castle's intrinsic factor" must be accepted with reservations, since the factors which govern the absorption of B_{12} in the normal rat must differ vastly from those which are operative in pernicious anemia.

The radioactivity tracer technique has been applied by Heinle and associates[29] to the study of the absorption and excretion of B_{12} in pernicious anemia. As a result of preliminary studies, these authors conclude that the intrinsic factor probably ". . . functions by increasing the utilization of orally administered vitamin B_{12}, and that failure of utilization in the absence of intrinsic factor is the result of failure of absorption."

Ungley,[30] while acknowledging that intrinsic factor is probably concerned with absorption of B_{12}, states: "It is unlikely, however, that the role of Castle's intrinsic factor is confined to absorption." In support of the hy-

[26] B. F. Chow, *Proc. Scient. Sessions 7th Ann. Meeting, Natl. Vitamin Found.* p. 1 (1952).

[27] B. F. Chow, C. Rosenblum, R. H. Silber, D. T. Woodbury, R. Yamamoto, and C. A. Lang, *Proc. Soc. Exptl. Biol. Med.* **76**, 393 (1951).

[28] O. D. Bird and B. Hoevet, *J. Biol. Chem.* **190**, 181 (1951).

[29] R. W. Heinle, A. D. Welch, V. Scharf, G. C. Meacham, and W. H. Prusoff, *Trans. Assoc. Am. Physicians* **65**, 214 (1952).

[30] C. C. Ungley, *Brit. J. Nutrition* **6**, 299 (1952).

pothesis that intrinsic factor may function within the tissues are the observations of Lajtha,[31] confirmed by Thompson[32] and by Franco and Arkun,[33] that free B_{12} added to marrow culture medium, unlike PGA, will not cause transformation, or "ripening," of megaloblasts into normoblasts. Callender and Lajtha[34] have subsequently shown that, whereas gastric juice when added by itself to such a marrow preparation has no ripening effect on megaloblasts, the combination of B_{12} and gastric juice will cause such elements to ripen into normoblasts. They interpret this observation as indicating that the hemopoietic activity of B_{12} is dependent upon the presence of the vitamin in a complex containing an "intrinsic factor." Gastric juice contains such a factor, and Ross has shown that, after parenteral administration to pernicious anemia patients, B_{12} is bound in a form which is unavailable to test organisms.[35] Since pernicious anemia serum, even with added free B_{12}, will not permit ripening of megaloblasts *in vitro*, the question arises as to why the free vitamin should be so highly efficacious when administered intravenously, or by other parenteral routes, to patients with pernicious anemia. Callender and Lajtha[34] attempt to explain this fact by suggesting that an extragastric intrinsic factor, not present in the free form in plasma, exists in the tissues of patients with pernicious anemia. They are in accord with the view that PGA (folic acid) or one of its derivatives acts directly on marrow cells but that, in pernicious anemia, such action is prevented by an inhibitor. The function of the inhibitor is counteracted by B_{12} when the latter is bound to gastric intrinsic factor in the case of marrow culture, or to a tissue factor when the vitamin is introduced *in vivo*. Evidence for the existence of such an inhibitor was afforded by earlier studies[31, 36] in which Lajtha and his associates showed that the serum of untreated pernicious anemia patients, in suitable concentrations, would not induce ripening of megaloblasts in tissue culture, but that after dilution, without the addition of any other factor, ripening of megaloblasts to normoblasts occurred. These findings have been confirmed by Thompson working in Ungley's laboratory.[32]

Whether through counteraction of an inhibitor or some other mechanism, the studies discussed above should provide at least a partial explanation for the observation of Horrigan *et al.*[37] that B_{12} instilled into the bone marrow of pernicious anemia patients causes transformation of megaloblastosis to normal erythropoiesis, whereas the instillation of PGA, and probably

[31] L. G. Lajtha, *Clin. Sci.* **9**, 287 (1950).
[32] R. B. Thompson, *Clin. Sci.* **9**, 281 (1950).
[33] J. Franco and S. N. Arkun, *Istanbul Contrib. Clin. Sci.* **1**, 92 (1951).
[34] S. T. Callender and L. G. Lajtha, *Blood* **6**, 1234 (1951).
[35] G. I. M. Ross, *Nature* **166**, 270 (1950).
[36] I. Rusznyák, S. Löwinger, and L. G. Lajtha, *Nature* **160**, 757 (1947).
[37] D. Horrigan, T. Jarrold, and R. W. Vilter, *J. Clin. Invest.* **30**, 31 (1951).

also CF, has no such direct effect. Girdwood[38] has found that the tissues of a patient dying with untreated pernicious anemia contain PGA or its conjugates, but he was unable to demonstrate the presence of B_{12}. The writer and his associates also have evidence that the marrow of pernicious anemia patients is not completely devoid of PGA or related compounds. It may, therefore, be inferred that B_{12} instilled directly into marrow promotes, in a manner not understood, but possibly through removal of an inhibitor effect, the metabolic function of PGA.

The therapeutic efficacy of PGA when given orally or parenterally in relatively large doses may be due to a mass action effect, as suggested by Vilter and associates.[39] In this important article the authors present theoretical considerations based on clinical evidence of the metabolic interrelationships of PGA, B_{12}, and other materials which participate, either as constituents or as enzymes, in the synthesis of nucleic acids, and they attempt to integrate earlier work on microbiological synthesis of nucleic acids with clinical observations on the therapeutic effects of PGA, B_{12}, thymine, and such precursor substances as uracil, methionine, and choline. In their view, megaloblastic erythropoiesis is the result of disturbed metabolism of nucleoprotein which normally depends upon a chain reaction involving the presence of methyl group donors, transmethylating agents, such as PGA and related compounds, and possibly unknown substances, such as the Will's factor. These agents enable purines and pyrimidines to be formed from precursor materials. B_{12} functions later in the chain reaction and is concerned with the synthesis of nucleosides, including thymidine. Supply of increased amounts of methyl donor compounds, namely methionine and choline, may, in certain circumstances, partially overcome a deficiency of PGA and produce hemopoietic response. Administration of PGA or CF will usually, if not always, cause at least temporary improvement, even in the presence of B_{12} deficiency, probably through a mass action effect in the presence of small quantities of B_{12}. The subsequent hematologic relapse and the frequent development or exacerbation of nervous system disease observed in pernicious anemia patients treated with PGA may be explained by depletion of remaining stores of B_{12}. It is of interest that the converse of this phenomenon has not been observed; that is, the administration of B_{12} to patients with primary PGA deficiency, such as in megaloblastic anemia of pregnancy, fails to produce even transient improvement and may actually serve to aggravate the clinical and hematologic manifestations due to lack of PGA. It must be supposed that in these situations

[38] R. H. Girdwood, *Edinburgh Med. J.* **58**, 309 (1951).

[39] R. W. Vilter, D. Horrigan, J. F. Mueller, T. Jarrold, C. F. Vilter, V. Hawkins, and A. Seaman, *Blood* **5**, 695 (1950).

administration of B_{12} accentuates the metabolic imbalance and leads to rapid exhaustion of whatever PGA compounds may remain.

Whereas the foregoing discussion includes much speculation and questionable analogies drawn between tissue culture phenomena, microbiological processes, and metabolic disturbances in man, the theories evolved have served to delineate the problems concerned with the metabolism of B_{12} and PGA and to emphasize the continuity and interdependence of their functions. Certainly there is no tendency on the part of active workers in this field to ignore newly discovered facts even though they may force modification or rejection of a favored hypothesis. There remains to be considered an aspect of the pathologic physiology of B_{12} to which reference has already been made; namely, the possible influence of injurious agents produced by intestinal bacteria. Before the discovery of the effect of liver on pernicious anemia the most favored etiologic explanation for this disease was intestinal sepsis with elaboration and absorption of hemolytic toxins. The introduction of liver therapy and the results of the clinical investigations of Castle and his group led to the concept of pernicious anemia as a conditioned nutritional deficiency disease, and the hemolytic component of the disorder was either disregarded or attributed to the lessened survival rate of defective erythrocytes. However, evidence has accumulated that potentially toxic agents may be present in the blood of patients with pernicious anemia and related anemias. Thus, in pernicious anemia in relapse there is a high blood phenol level and an increased urinary excretion of phenolic compounds.[40] Liver extract or desiccated stomach will correct the abnormal excretion of phenols by a reduction in the output of the fraction containing the hydroxyphenyl acids, and more recently it has been shown that B_{12} has the same effect.[41] Since the hydroxyphenyl acids are metabolic intermediates derived from tyrosine, it seems probable that tyrosine or a tyrosine derivative plays a part in erythropoiesis. If the accumulated products of tyrosine breakdown are toxic to circulating erythrocytes, which has not been demonstrated, then one of the therapeutic actions of B_{12} might be the arrest of excessive red cell breakdown through its effect on tyrosine metabolism. This concept, which is highly speculative, is in accord with the well-established fact that institution of adequate treatment of pernicious anemia with B_{12}, liver extract, or desiccated stomach is followed within a few days by reduction in hemolysis and disappearance of clinical evidences of toxicity, including fever and anorexia.

On the other hand, hemolysis and other toxic manifestations in pernicious anemia may be due to circulating substances of intestinal bacterial

[40] M. E. Swendseid, B. Wandruff, and F. H. Bethell, *J. Lab. Clin. Med.* **32**, 1242 (1947).

[41] L. D. Abbot, Jr., and G. W. James, III, *J. Lab. Clin. Med.* **35**, 35 (1950).

origin. It has been shown that indol has a hemolytic effect in dogs maintained on a diet deficient in B vitamins but not in dogs on an adequate diet.[42] The report of the therapeutic efficacy of aureomycin in pernicious anemia[43] has been confirmed by the writer and his associates, who have also obtained responses to the administration of terramycin and chloramphenicol. The effects of these agents are slow and prolonged and may be enhanced by the supplemental oral administration of B_{12} in small doses such as 2 to 5 γ daily.

The action of antibiotics in pernicious anemia may be due either to the elimination of organisms which require B_{12} for growth and so deprive the host of the vitamin or to the removal of bacteria which elaborate the postulated injurious substance(s). The latter explanation has some indirect evidence in its support, since the mechanism of injury associated with such bacterial products may include both hemolysis of circulating red cells and inhibition of normal erythropoiesis. A hemolytic effect of plasma from untreated pernicious anemia patients has been reported by De Gowin and associates,[44] and reference has been made to the existence of an erythropoietic inhibitor in the serum of such patients. In the present state of our knowledge, it seems probable that whatever injurious influences may be present in pernicious anemia are counteracted by B_{12} and that the action of antibiotics, in inducing remission, is concerned chiefly with making B_{12} available to the organism. It should be mentioned, however, that an alternative explanation of the effect of antibiotics in pernicious anemia has been suggested as a possibility by Lichtman and associates[43] and is favored by Welch and Heinle.[45] In their view, such drugs as aureomycin probably induce hemopoietic responses by producing alterations in intestinal flora with the establishment of species which synthesize and release significant amounts of readily absorbable PGA or related compounds. Welch and Heinle cite the observations of Foy and associates[46] on the effectiveness of penicillin in a case of megaloblastic anemia of pregnancy as evidence in support of their contention that antibiotics operate by making PGA rather than B_{12} available. There is, however, no evidence that PGA is produced in greater abundance or is absorbed more efficiently in the alimentary tract of patients who are receiving antibiotics.

In the foregoing discussion B_{12} has been identified as the dietary extrinsic factor of Castle as well as the substance in liver which is chiefly, if

[42] C. P. Rhoads, W. H. Barker, and D. K. Miller, *J. Exptl. Med.* **67**, 299 (1938).

[43] H. Lichtman, V. Ginsberg, and J. Watson, *Proc. Soc. Exptl. Biol. Med.* **74**, 884 (1950).

[44] E. L. De Gowin, H. E. Hamilton, R. F. Sheets, C. D. Janney, and J. A. Ellis, *J. Lab. Clin. Med.* **40**, 790 (1952).

[45] A. D. Welch and R. W. Heinle, *Pharm. Revs.* **3**, 345 (1951).

[46] H. Foy, A. Kondi, and A. Hargreaves, *Brit. Med. J.* **I**, 1108 (1951).

not entirely responsible, for the therapeutic efficacy of parenterally administered liver extracts in pernicious anemia. By implication at least, the conclusion has been drawn that pernicious anemia is a B_{12} deficiency state, induced by a gastric abnormality, and that whatever other nutritional factors or toxic agents may be operative are either the direct result of lack of B_{12} or, in any event, are counteracted completely by supplying B_{12} to the tissues. The presence in liver of PGA, or more probably its formyl derivative CF,[47] is, or course, well known and presumably accounts for the efficacy of this material in PGA deficiency states, such as megaloblastic anemia of pregnancy. The responses obtained in pernicious anemia to the oral administration of whole liver or liver extracts may be attributable to PGA, perhaps augmented by absorption of some of the B_{12} present in the liver. It is also entirely possible that there are other as yet unidentified hemopoietic factors in liver. In the manufacture of refined liver extracts of high potency almost all the PGA compounds are eliminated in the process of fractionation, and the therapeutic efficacy of such preparations can be entirely accounted for on the basis of their B_{12} content.[48]

Most clinical observers are in agreement that crystalline B_{12}, or concentrates of the vitamin obtained from cultures of streptomyces (*Str. griseus*, *Str. aureofaciens*), are fully as effective as liver extracts in the treatment of pernicious anemia, and, in fact, U. S. P. liver injection preparations are now standardized by B_{12} assay rather than by clinical response tests.[49] The report of Beard and associates[50, 51] that B_{12} failed to maintain normal hematologic values in approximately half of a group of twenty-seven patients after 5 to 10 months of treatment is probably explained, as suggested by the authors, by the poor diets of the patients, particularly with respect to sources of good-quality protein. The importance of animal protein in hemopoiesis and a correlation between macrocytic anemia and low intake of protein of good biologic quality during pregnancy have been previously reported.[52] Other statements in the literature to the effect that B_{12} may not correct all the hematologic values in pernicious anemia, even with the addition of PGA, are of interest but have not been confirmed, possibly because of the highly specialized techniques employed by those who claimed to have demonstrated them. Thus Larsen[53] reported that erythrocytes of normal volume but increased diameter and lessened thickness are present in the

[47] M. E. Swendseid, F. H. Bethell, and W. W. Ackermann, *J. Biol. Chem.* **190**, 791 (1951).

[48] R. H. Girdwood, K. M. Carmichael, and B. Woolf, *Brit. Med. J.* **II**, 1357 (1950).

[49] U. S. Pharmacopeia, 14th revision, 3rd Supplement, 1951.

[50] M. F. Beard, S. K. McIlvanie, and M. Nataro, *Southern Med. J.* **43**, 678 (1950).

[51] M. F. Beard and S. K. McIlvanie, *Proc. 3rd Intern. Congr. Haematol.* Cambridge, England, 1950, Grune & Stratton, New York, p. 34, 1951.

[52] F. H. Bethell, E. Blecha, and J. H. Van Sant, *J. Am. Dietet. Assoc.* **19**, 165 (1943).

[53] G. Larsen, *Proc. 3rd Intern. Congr. Haematol.* Cambridge, England, 1950, Grune & Stratton, New York, p. 25, 1951.

blood of pernicious anemia patients under treatment with B_{12}, purified liver extracts, or PGA. The change in the physical properties of the cells was attributed to the presence of an abnormal form of hemoglobin. Owren[54-56] supports Larsen's hypothesis concerning such abnormal hemoglobin and believes that the defect lies in the protein rather than the heme portion of the molecule. The metabolic dysfunction, which may be corrected by crude liver extracts but not, according to Owren, by purified liver extracts, B_{12}, or PGA, involves protein synthesis within the liver and results not only in the production of an abnormal hemoglobin but also in a decreased formation of prothrombin as measured by Owren's technique.

Whether or not the claims of Larsen and Owren with respect to the superiority of crude liver extracts over purified extracts and B_{12} ultimately receive support from other investigators, the fact remains that purified liver extracts have been used extensively in the treatment of pernicious anemia ever since their introduction in the mid-nineteen thirties, and the concensus of almost all clinicians is that such preparations provide complete remission as evidenced by relief of clinical manifestations and restoration to normal of all of the usually determined hematologic values. In the words of Wm. P. Murphy, "There is much evidence available to confirm the beneficial effect of the concentrated extracts on all of the disturbances characteristic of pernicious anemia including those due to neural damage. No evidence has been presented to show that 'crude' extracts are more beneficial in any respect."[57]

Of the several analogs of vitamin B_{12} which have been isolated and made available for clinical trial, none has been shown to differ in therapeutic properties or degree of effectiveness from B_{12} itself (cyanocobalamin).[12, 58-63] However, it seems likely that quantitative differences in potency among the cobalamin analogs will be detected as their use continues.

4. Fish Tapeworm Infestation

Megaloblastic anemia associated with fish tapeworm (*Diphyllobothrium latum*) infestation has been shown to be a true B_{12} deficiency state. Earlier,

[54] P. A. Owren, *Scand. J. Clin. Lab. Invest.* **2**, 241 (1950).
[55] P. A. Owren, *Proc. 3rd Intern. Congr. Haematol.* Cambridge, England, 1950, Grune & Stratton, New York, p. 22, 1951.
[56] P. A. Owren, *Proc. 3rd Intern. Congr. Haematol.* Cambridge, England, 1950, Grune & Stratton, New York, p. 34, 1951.
[57] W. P. Murphy, *Blood* **3**, 32 (1948).
[58] H. Lichtman, J. Watson, V. Ginsberg, J. V. Pierce, E. L. R. Stokstad, and T. H. Jukes, *Proc. Soc. Exptl. Biol. Med.* **72**, 643 (1949).
[59] R. F. Schilling, J. W. Harris, and W. B. Castle, *Blood* **6**, 228 (1951).
[60] C. C. Ungley and H. Campbell, *Brit. Med. J.* **I**, 152 (1951).
[61] J. N. M. Chalmers, *Brit. Med J.* **I**, 161 (1951).
[62] G. C. K. Reid, *Brit. Med. J.* **I**, 164 (1951).
[63] G. W. James, III, and L. D. Abbott, Jr., *Proc. Soc. Exptl. Biol. Med.* **77**, 416 (1951).

before B_{12} had been isolated and identified as extrinsic factor, von Bonsdorff[64] had shown that for anemia to occur in fish tapeworm infestation it was necessary that the worm be lodged in the upper portion of the small intestine. He believed that the worm interfered with the interaction of intrinsic and extrinsic factors, and he made the shrewd observation: "Much confusion in the discussions could, I believe, be avoided if the ability of the worm to produce pernicious anemia was consistently kept separate from its other toxic properties." Later studies have established the validity of this statement, since von Bonsdorff and his colleagues[65, 66] have demonstrated the presence of B_{12} in considerable amounts in dried fish tapeworm. They obtained therapeutic responses in addisonian pernicious anemia patients who were given the dried worm as a source of extrinsic factor (B_{12}) in combination with normal gastric juice containing intrinsic factor, as well as in patients with fish tapeworm anemia who received the dried worm without a source of intrinsic factor. These observations are interpreted as indicating that the worm, if located sufficiently high in the intestine, may utilize the naturally available B_{12} even in the presence of intrinsic factor and so lead to B_{12} deficiency in the host. If B_{12} is administered orally in amounts greater than the worm can assimilate, the deficiency will be overcome.

5. Gastrectomy

Megaloblastic anemia following total gastrectomy may be attributed, at least in part, to removal of the source of supply of intrinsic factor. However, other digestive disturbances affecting nutrition appear to be of importance in some cases. Thus, some patients who develop macrocytic anemia after removal of the stomach can be maintained in satisfactory remission for long periods by the parenteral administration of purified liver extract or B_{12} (author's cases) whereas others fail to regain fully normal hematologic values or exhibit a fall in values after good initial responses. There is evidence that deficiency of PGA, not relieved by B_{12}, may be present after gastrectomy.[67]

6. Intestinal Abnormalities

In megaloblastic anemia associated with intestinal strictures or anastomoses, the therapeutic response to B_{12} has been variable, and it appears that PGA rather than B_{12} deficiency may be largely responsible for the development of anemia in these cases. Ungley[68] reported two such cases

[64] B. von Bonsdorff, *Blood* **3**, 91 (1948).
[65] B. von Bonsdorff and R. Gordin, *Acta Med. Scand.* **140**, *Suppl.* **259**, 112 (1951).
[66] B. von Bonsdorff and R. Gordin, *Acta Med. Scand.* **142**, *Suppl.* **263**, 283 (1952).
[67] N. S. Conway and H. Conway, *Brit. Med. J.* **I**, 158 (1951).
[68] C. C. Ungley, *Proc. Roy. Soc. Med.* **43**, 537 (1950).

in which the responses obtained following the injection of 80 γ of B_{12} were suboptimal. One of these patients was subsequently maintained in remission by the oral administration of 100 γ daily.

7. Human Growth Factor

Vitamin B_{12} dietary supplementation was followed by promotion of growth in children whose growth rates were below standard, as measured by Wetzel grid records. The effect of orally administered B_{12} was first evaluated by Wetzel and associates[69] in a small group of institutionalized children. They reported substantial growth gains in 5 of 11 children with retarded growth. A later study was conducted by Wetzel and others[70] on school children of a high-income suburban community. They found that, in spite of their favorable economic situation and freedom from evidences of malnutrition or chronic illness, one-third of the entire school population were in relative growth failure. Vitamin B_{12} in daily oral doses of 10 γ was administered to 36 of the 40 children with retarded growth rates. Four of the group were untreated on their own volition. No changes in diet were made in any of the children. Significant growth responses were obtained in 23 of the 36 treated children. The 4 untreated children showed, on successive observations, aggravation of their growth failure. It was recorded that retarded growth was accompanied by impaired academic achievement, substandard physical performance, and increased fatiguability. Rapid improvement in all of these deficiencies was observed in children whose growth failure was corrected by B_{12} supplementation.

The observations of Wetzel and his coworkers are of great interest but require confirmation and amplification by others before the conclusions drawn from these studies can be fully accepted. The grid method of recording physical development and fitness, devised by Wetzel, probably provides the most precise measurement of growth rates which is available, but the great number of variables which influence human growth render an adequately controlled and objective study of this nature exceedingly difficult. Further investigations should be carried out with particular reference to dietary analyses of the subjects, random selection of B_{12} recipients and controls, and provisions to avoid subjective bias on the part of those conducting the tests as a possible determinant in the results.

8. Neurologic Disorders

Painful neuropathies associated with a miscellaneous group of conditions including malnutrition, chronic alcoholism, diabetes, tabes dorsalis, and

[69] N. C. Wetzel, W. C. Fargo, I. H. Smith, and J. Helikson, *Science* **110**, 651 (1949).
[70] N. C. Wetzel, H. H. Hopwood, M. E. Kuechle, and R. M. Grueninger, *J. Clin. Nutrition* **1**, 17 (1952).

trigeminal neuralgia have been reportedly benefited by the administration of B_{12}.[71-75] The favorable effect of B_{12} on the neurologic lesions of pernicious anemia was undoubtedly a major factor in leading to its use in other nervous system disorders. However, it should be pointed out that in these conditions of obscure etiology and therapeutic refractoriness a great variety of agents have temporarily enjoyed enthusiastic acceptance. The parenteral doses of B_{12} for which effectiveness has been claimed have varied from 15 γ to as much as 1000 γ. In general, relatively massive doses have been employed. Since amounts in the order of 1000 γ are far beyond the range of dosage required for replacement therapy, any particular effect obtained from them is probably attributable to a pharmacologic action rather than to the supply of a deficient nutrient.

It seems entirely possible that lack of B_{12} may be a component of complex deficiency states associated with peripheral neuropathy. However, by analogy with pernicious anemia, it is difficult to explain the rarity of subacute combined degeneration of the spinal cord in such nutritional disorders if their neurologic manifestations are due in any large part to B_{12} deficiency. The function of B_{12} in the maintenance of the integrity of the neurones is not understood, and its therapeutic role in various neuropathies, other than that associated with pernicious anemia, has not been clearly defined.

9. Summary

In summary, the major manifestation of B_{12} deficiency in man is anemia characterized by an abnormal, or megaloblastic, type of erythropoiesis. Concurrently, alterations in leukopoiesis and thrombocyte production occur. The hematologic changes associated with a lack of B_{12} are identical with those observed in PGA deficiency states, and there is evidence to indicate that the function of B_{12} in hemopoiesis is mediated through its effect on the metabolism of PGA and related compounds.

Vitamin B_{12}, including some of its analogs, is identical with the extrinsic factor of Castle. In pernicious anemia, deficiency of the vitamin results from the absence of Castle's intrinsic factor, which is secreted by the normal stomach. Intrinsic factor may act by combining stoichiometrically with B_{12} to form an unstable complex which protects the vitamin from utilization by bacteria or its destruction in some other manner within the intestinal tract. An intrinsic factor present in the tissues may also function in the normal metabolism of B_{12}. Furthermore, there is evidence that in pernicious anemia, as a result of B_{12} deficiency, toxic or inhibiting agents

[71] W. B. Bean, M. Franklin, and A. L. Sahs, *Am. J. Med. Sci.* **220**, 431 (1950).
[72] S. M. Sancetta, P. R. Ayres, and R. W. Scott, *Ann. Internal. Med.* **35**, 1028 (1951).
[73] W. S. Fields and H. E. Hoff, *Neurology* **2**, 131 (1952).
[74] W. S. Fields and H. E. Hoff, *Merck Rept.* October, 1952.
[75] J. Lereboullet and R. Pluvinage, *J. Am. Med. Assoc.* **148**, 667 (1952).

may gain entrance to the body. Such agents may be of intestinal bacterial origin. The therapeutic effect of B_{12} on the neurologic lesions of pernicious anemia, which is not exerted by PGA, suggests that B_{12} is concerned with metabolic processes affecting the integrity of neurones.

The effectiveness in pernicious anemia of refined and concentrated liver extracts appears to depend entirely on their content of B_{12} (cyanocobalamin) and its analogs, such as B_{12b} (hydroxocobalamin). The claims of some investigators that B_{12} constitutes incomplete treatment of pernicious anemia and that superior results are obtained from the use of crude liver extracts have not been substantiated and are counter to the accumulated experience of a great many clinical observers.

The megaloblastic anemia associated with some cases of *Diphyllobothrium latum* infestation is apparently due to diversion of B_{12} in the alimentary tract from the host to the nutritional requirements of the tapeworm. Anemia following gastrectomy is presumably in part the result of elimination of the source of intrinsic factor, but other disturbances of digestion or absorption are undoubtedly factors in its production. Intestinal anastomoses, strictures, and blind loops cause complex nutritional disturbances which depend upon diminished absorptive surface, altered motility, stasis and changes in bacterial flora. Megaloblastic anemia is of common occurrence in such situations and may result from deficiencies of both B_{12} and PGA in varying degrees.

Observations on the effect of B_{12} administration to children with retarded physical development have indicated that the vitamin may have growth-promoting properties even though the diets of such children have not been obviously inadequate. It has been proposed that in such cases B_{12} supplementation may correct a variety of metabolic derangements which are collectively responsible for the slow-down in the rate of growth. Additional careful nutritional studies will have to be carried out before the role of B_{12} as a growth-promoting agent in children can be adequately evaluated.

Therapeutic success has been claimed for B_{12} in relieving sensory disturbances in a number of neurologic disorders other than pernicious anemia. Although deficiency of B_{12} may be a factor in some nutritional disorders associated with neuropathy, it would appear that in most of the conditions treated there is no evidence for the existence of such deficiency. Whatever benefit follows the use of massive doses of the vitamin must be attributed to a pharmacologic action rather than to correction of a metabolic defect.

XII. Pharmacology

FRANK H. BETHELL

A. ACTION AND USES

Vitamin B_{12} is probably concerned with a wide variety of metabolic processes which include factors affecting growth, hemopoiesis, and the maintenance of the integrity of nerve cells. The primary therapeutic use of B_{12} is in the treatment of pernicious anemia and certain related megaloblastic anemias which are due to deficiency of the vitamin. B_{12} has also been employed as a growth-promoting agent in children with retarded physical development, and promising results have followed its use in a variety of neurologic disorders characterized by painful peripheral neuropathy.

B. DOSE

In pernicious anemia the usual dose is 10 to 30 γ by intramuscular injection daily or thrice weekly during the initial period of treatment and at intervals of 1 to 3 weeks for maintenance of remission.[1]

Oral B_{12} therapy in pernicious anemia is relatively ineffective and unreliable unless a source of intrinsic factor of potency proved by clinical trial is administered together with the vitamin.[2]

Nutritional megaloblastic anemia due to B_{12} deficiency may be effectively treated by the daily oral administration of 10 to 50 γ without a source of intrinsic factor. In most complex deficiency states associated with megaloblastic anemia, the use of both vitamin B_{12} and pteroylglutamic acid is indicated.[3]

As a dietary supplement to promote growth in retarded children, 10 γ daily has been employed.[4]

In the treatment of neuropathies other than pernicious anemia, a wide range of doses has been used, but best results have been reported following the parenteral administration of 500 to 1000 γ, which can be repeated without ill effect at frequent intervals.[5]

C. TOXICITY

No acute or chronic toxic effects have been observed even after the parenteral administration of very large doses of B_{12}.

[1] F. H. Bethell, *Wisconsin Med. J.* **51**, 1082 (1952).

[2] J. W. R. Everse, K. W. Thompson, H. G. Wijmenga, and R. Wolff, *Blood* (In press).

[3] F. H. Bethell, M. E. Swendseid, S. Miller, and A. A. Cintron-Rivera, *Ann. Internal Med.* **35**, 518 (1951).

[4] N. C. Wetzel, W. C. Fargo, I. H. Smith, and J. Helikson, *Science* **110**, 651 (1949).

[5] W. S. Fields and H. E. Hoff, *Merck Rept.*, October, 1952.

XIII. Requirements

THOMAS H. JUKES and WILLIAM L. WILLIAMS

The vitamin B_{12} requirement of most animals which have been studied appears to be in the neighborhood of 10 γ per kilogram of diet. The requirement is increased by raising the protein content of the diet, by hyperthyroidism including the feeding of thyroxine, and by reproductive activities in the female. Vitamin B_{12} is unusual in two respects. First, the apparent requirement may be markedly influenced in human beings by a degenerative process in the gastric mucosa which diminishes the uptake of the vitamin from the gut, thus necessitating either the oral administration of massive doses, or the injection of the vitamin, or the feeding of preparations of the "intrinsic factor." Second, a dietary deficiency of cobalt in ruminating animals apparently leads to a diminution of the production of vitamin B_{12} by microorganisms in the paunch, thus resulting in vitamin B_{12} deficiency in the animal.

The information regarding vitamin B_{12} in the nutrition of certain animals is summarized in the following pages.

A. OF CHICKS

THOMAS H. JUKES and WILLIAM L. WILLIAMS

The nutritional requirements of chicks for rapid growth and good hatchability are marked and exacting. The history of the successful development of the poultry industry is interwoven with the discovery of certain vitamins and their use in feeds. As knowledge of the vitamins expanded, it appeared that certain animal protein feedstuffs contained a factor which was needed for hatchability and growth and that this factor differed from riboflavin.[1-5] Research in this field was stimulated by the occurrence of a shortage of animal proteins, especially fish meal and milk, which was occasioned by wartime conditions. Simultaneously the rate of commercial production of chickens for meat increased and it became necessary to use larger proportions of two of the most readily available feedstuffs, corn and soybeans. These two feedstuffs are deficient in vitamin B_{12}, and many investigations were involved with circumstances arising from this fact.

Investigations by Hammond[6] showed that cow manure or dried rumen

[1] J. B. Christiansen, H. J. Deobald, J. G. Halpin, and E. B. Hart, *Poultry Sci.* **19**, 18 (1940).

[2] J. S. Carver and R. J. Evans, *U. S. Egg Poultry Mag.* **49**, 468 (1943).

[3] T. C. Byerly, H. W. Titus, N. R. Ellis, and R. B. Nestler, *Poultry Sci.* **16**, 322 (1937).

[4] R. B. Nestler, T. C. Byerly, N. R. Ellis, and H. W. Titus, *Poultry Sci.* **15**, 67 (1936).

[5] W. W. Cravens, W. H. McGibbon, and J. G. Halpin, *Poultry Sci.* **24**, 305 (1945).

[6] J. C. Hammond, *Poultry Sci.* **21**, 554 (1942); **23**, 358, 471 (1944).

contents had a marked effect in improving the growth of chicks and turkeys when added to diets consisting principally of ground wheat, soybean meal, and alfalfa meal. Studies by Hammond and Titus[7] indicated that on similar diets the early growth of chicks was improved by adding 2% of sardine fish meal to the basal mixture.

At first it was suggested that androgens and riboflavin were the factors principally involved in the growth-promoting effects of cow manure, but later it became apparent that an unknown vitamin-like substance, the "cow manure factor," was largely responsible for remedying the deficiency which existed in the vegetable diet. Thus was foreshadowed the eventual development which led to the industrial production of vitamin B_{12} by microbial fermentation, for it is the microflora of the digestive tract of the cow which lead to the presence of the vitamin in the feces and rumen contents.

The investigations started by Hammond and Titus were continued by Rubin and Bird,[8] who prepared concentrates of the cow manure factor and carried out experiments which further differentiated it from the known vitamins including folic acid and vitamin B_6. The factor was concentrated from cow manure to some extent by extraction with acid and precipitation with ammonium sulfate. The Beltsville group explored widely the nutritional relationships of the factor as regards growth and hatchability. It was shown that a deficiency of the factor as measured by hatchability of eggs or by chick growth was accentuated by increasing the soybean meal content of the diet.[8a] This observation provided a point of similarity with the factor X of Cary and Hartman, the requirement for which had been shown to be increased by adding soybean meal or alcohol-extracted casein to the basal diet. The use of a diet containing 70% of soybean meal made it more easily possible to produce the deficiency experimentally in chicks. However, an even more important consideration was found to be the carry-over of the factor from the maternal diet through the egg into the chick. When hens received diets containing from 5 to 10% of fish meal, their offspring were found to grow quite rapidly on all-vegetable diets. In contrast, when hens were placed on all-vegetable diets the hatchability of their eggs tended to diminish, and the chicks which hatched grew slowly on the basal diet unless they were supplied with a dietary source of the factor.

Bethke and coworkers[9, 10] found that a diet in which soybean meal was the only source of supplemental protein was adequate for egg production but lacked a factor for hatchability which was present in pork liver, liver extract, fish solubles and, to a lesser extent, meat scraps. When chicks

[7] J. C. Hammond and H. W. Titus, *Poultry Sci.* **23**, 49 (1944).
[8] M. Rubin and H. R. Bird, *J. Biol. Chem.* **163**, 393 (1946).
[8a] D. Whitson, H. W. Titus, and H. R. Bird, *Poultry Sci.* **25**, 52 (1946).
[9] R. M. Bethke, D. C. Kennard, and V. D. Chamberlin, *Poultry Sci.* **25**, 589 (1946).
[10] R. M. Bethke, J. M. Pensack, and D. C. Kennard, *Poultry Sci.* **26**, 128 (1947).

hatched from eggs laid by hens on various supplements were placed on the basal corn-soybean meal diet, it was evident that a growth-promoting factor in fish products was transmitted from the hen through the egg to the chick. These results are illustrated in Table XIX.

The necessity for a dietary supply of the factor for the production of hatchable eggs provided a clue to the reason for a seasonal variation in hatchability which was described by the Beltsville group.[3, 11] The factor was shown to be present not only in cow manure but also in chicken manure,[12]

TABLE XIX

EFFECTS OF FISH MEAL IN THE DIETS OF HENS AND CHICKS UPON THE GROWTH OF CHICKS[10]

Diets		Trial	Number of chicks	Average weight, g.			Mortality, %
Hens	Chicks			0 wk.	2 wk.	6 wk.	
Soybean oil meal	Soybean oil meal	1	53	42.7	93.0	321.6	1.9
		2	39	40.3	95.8	318.0	2.5
	Soybean oil meal plus 4% fish meal	1	51	42.7	116.0	472.1	5.9
		2	38	40.3	112.8	472.5	5.3
Soybean oil meal plus 2% fish solubles or fish meal	Soybean oil meal	1	50	42.7	115.6	401.8	0.0
		2	50	39.9	110.6	413.0	0.0
	Soybean oil meal plus 4% fish meal	1	50	42.7	125.1	501.8	2.0
		2	50	39.9	117.6	462.9	2.0

and in the latter case bacterial production of the factor took place after the feces had been voided. Furthermore, in cold weather the fermentation which led to the biogenesis of the factor was evidently slowed markedly so that insufficient quantities were ingested by the hens during their alimentary explorations of the litter on the floor of their houses. With the advent of warmer weather, fermentation in the litter was accelerated and the hens then obtained enough of the factor from their excreta to enable hatchable eggs to be produced. The fecal matter was also shown to be capable of producing a growth response in chicks on the all-vegetable diet.[12-14]

[11] D. Whitson, H. W. Titus, and H. R. Bird, *Poultry Sci.* **25**, 143 (1946).
[12] M. Rubin, H. R. Bird, and I. Rothchild, *Poultry Sci.* **25**, 526 (1946).
[13] J. McGinnis, J. M. Stevens, and K. Groves, *Poultry Sci.* **26**, 432 (1947).
[14] A. C. Groschke, M. Rubin, and H. R. Bird, *Poultry Sci.* **27**, 302 (1948).

Meanwhile a close relationship had become evident between the anti-pernicious anemia factor of concentrated liver extracts and the factor needed by chicks on vegetable diets. The evidence was as follows:

1. The chick factor ("animal protein factor") had points of similarity to factor X, needed by rats, which was also present in crude animal proteins and absent from vegetable proteins. Furthermore, deficiencies of either factor X in rats or the animal protein factor in chicks were intensified by depleting the maternal supply or by raising the level of vegetable protein in the basal diet. In 1946, it was shown by Cary *et al.*[15] that concentrated injectable liver extract, 15 U.S.P. units per cubic centimeter, was a highly potent source of factor X and that by precipitation with ammonium sulfate a fraction could be prepared from the extract which was effective in amounts of a few micrograms. Ammonium sulfate had been reported to precipitate the anti-pernicious anemia factor.[16]

TABLE XX

Activity of Anti-Pernicious Anemia Preparations in Chicks[17]

Basal diet: Corn, soybean meal, wheat bran, wheat middlings, casein, alfalfa meal, minerals, and pure vitamins

Experiment	Supplement	Average weight at 4 weeks
1	None	219
1	3% Lederle liver powder	259
1	0.1 ml. Reticulogen (Lilly) daily by mouth	262
2	None	209
2	0.05 ml. Reticulogen daily by intramuscular injection	301

2. Anti-pernicious anemia liver extracts were found to be good sources of the animal protein factor in tests with chicks (Table XX).

In the spring of 1948, the crystallization of the anti-pernicious anemia factor (vitamin B_{12}) from liver was reported.[18, 19] The vitamin was soon found to have biological properties corresponding to those which had been previously established for concentrates of the animal protein factor.

It was reported in July 1948 that remission in pernicious anemia was produced by injecting concentrates of the animal protein factor, standard-

[15] C. A. Cary, A. M. Hartman, L. P. Dryden, and G. D. Likely, *Federation Proc.* **5**, 128 (1946).

[16] H. D. Dakin, C. C. Ungley, and R. West, *J. Biol. Chem.* **115**, 771 (1936).

[17] C. A. Nichol, A. R. Robblee, W. W. Cravens, and C. A. Elvehjem, *J. Biol. Chem.* **170**, 419 (1947).

[17a] J. E. Ford, S. K. Kon, and J. W. G. Porter, *Biochem. J.* **50**, ix (1951).

[18] E. L. Rickes, N. G. Brink, F. R. Koniuszy, T. R. Wood, and K. Folkers, *Science* **107**, 396 (1948).

[19] E. Lester Smith, *Nature* **161**, 638 (1948); **162**, 144 (1948).

ized by chick assay, which were prepared from cultures of a bacterial organism isolated from hen feces.[20] Also in July 1948 it was reported that vitamin B_{12} was effective at low levels in promoting growth in chicks on a corn-soybean meal basal diet.[21] These experiments showed that a crystalline substance, vitamin B_{12}, had biological properties corresponding both to those of the anti-perinicious anemia factor of concentrated liver extracts and to the animal protein factor which was needed by chicks. Shortly thereafter it was demonstrated that vitamin B_{12} was active in promoting growth in thyroid-fed rats[22] and that vitamin B_{12} could replace factor X for rats.[23] The requirement of chicks was found to be in the neighborhood of 10 to 20 γ of the crystalline substance per kilogram of diet. This corresponded closely to the amount calculated from experiments with liver extract, since from 1 to 2 γ of vitamin B_{12} when injected into a patient with pernicious anemia produced a hemopoietic response corresponding to about 1 U.S.P. unit, and about 0.5 to 1.0 ml. of concentrated liver extract, 15 units per milliliter was found to provide adequate supplementation for chicks when added to 1 kg. of corn-soybean meal diet.[24] It was possible to compare the effectiveness of oral and injected doses; about 0.37 γ when fed by pipet weekly could be calculated to give a response corresponding to 0.2 γ injected intramuscularly. The requirement for vitamin B_{12} by the oral and injected routes in normal chicks affords an interesting contrast with the requirements for pernicious anemia patients, if an arbitrary figure of 50 kilograms is taken from the body weight of such patients:

	Approximate daily requirement of vitamin B_{12} per kilogram of body weight	
	Injected	Oral[a]
Pernicious anemia patients	0.02–0.04 γ	40–150 times the minimum parenteral dose[1, 25]
Chicks	0.2 γ	0.4 γ[21]

[a] Without supplemental intrinsic factor.

These calculations reveal that the pernicious anemia patient has a *much smaller* requirement per kilogram of body weight than normal chicks for

[20] E. L. R. Stokstad, A. C. Page, Jr., J. V. Pierce, A. L. Franklin, T. H. Jukes, R. W. Heinle, M. Epstein, and A. D. Welch, *J. Lab. Clin. Med.* **33**, 860 (1948).

[21] W. H. Ott, E. L. Rickes, and T. R. Wood, *J. Biol. Chem.* **174**, 1047 (1948).

[22] G. A. Emerson, *Proc. Soc. Exptl. Biol. Med.* **70**, 392 (1949).

[23] A. M. Hartman, L. P. Dryden, and C. A. Cary, *J. Am. Dietet. Assoc.* **25**, 929 (1949).

[24] E. L. R. Stokstad, T. H. Jukes, J. V. Pierce, A. C. Page, Jr., and A. L. Franklin, *J. Biol. Chem.* **180**, 647 (1949).

[24a] C. C. Ungley, *Brit. Med. J.* **2**, 905 (1950).

[25] L. M. Meyer, A. Sawitsky, B. S. Cohen, M. Krim, and R. Fadern, *Am. J. Med. Sci.* **220**, 604 (1950).

injected vitamin B_{12}. The requirement of rats on a diet containing thyroid powder is even higher than that of the chick. Ruminants also have comparatively high requirements. This was not realized at first, and cobalt-deficient ruminants were dosed with amounts of vitamin B_{12} based on the levels used in patients with pernicious anemia; these amounts were insufficient for the animals (p. 521).

Vitamin B_{12} was found to improve the hatchability of eggs when injected into hens[26] which were kept on all-vegetable diets. The amount needed for good results, including sufficient carry-over to permit good growth of the chicks, was about 4 γ per week.

In a search for new sources of the vitamin for use in poultry feeds, it was found that cultures of *Streptomyces aureofaciens* supplied not only vitamin B_{12} but also a new growth factor which was not identical with any of the known vitamins. This factor was identified as aureomycin, and other antibiotics were shown to produce similar effects in increasing the growth rate of chicks fed supposedly adequate diets.[27]

TABLE XXI

PANTOTHENIC ACID CONTENT OF CHICK LIVERS AS AFFECTED BY DIETARY VITAMIN B_{12}[28]

Group No.	Supplement	Pantothenic acid content of liver tissue	
		Mylase-P digestion, γ/g.	Phosphatase and chicken liver enzyme digestion, γ/g.
1	None	150	153
2	Vitamin mixture[a]	147	144
3	Vitamin B_{12}, 20 γ/kg. of diet	56	54
4	Vitamin mixture plus B_{12}	61	60

[a] Thiamine, calcium pantothenate, pyridoxine, folic acid, and biotin.

1. RELATIONSHIPS BETWEEN PANTOTHENIC ACID AND VITAMIN B_{12} IN CHICKS

Evans and coworkers[28] found that when chicks were fed vitamin B_{12} the apparent pantothenic acid content of their liver tissue was much lower than that of the liver tissue of chicks fed the unsupplemented vitamin B_{12}-deficient diet. Pantothenic acid was assayed with *L. arabinosus* after digestion with either Mylase-P or intestinal phosphatase and chicken liver

[26] C. F. Petersen, A. C. Wiese, C. E. Lampman, and R. V. Dahlstrom, *Poultry Sci.* **29**, 618 (1950).

[27] E. L. R. Stokstad, and T. H. Jukes, *Proc. Soc. Exptl. Biol. Med.* **73**, 523 (1950).

[28] R. J. Evans, A. C. Groschke, and H. A. Butts, *Arch. Biochem.* **31**, 454 (1951).

enzyme. Some of the results are shown in Table XXI. The authors suggested that vitamin B_{12} aids in the transfer of pantothenic acid from the liver for use elsewhere in the body of the chick. Similar experimental results were reported by Yacowitz and coworkers,[29] who also noted that the free pantothenic acid content of breast muscle tissue was not affected by dietary vitamin B_{12} while the free pantothenate content of liver tissue was decreased as compared with the content of the livers of chicks receiving no dietary vitamin B_{12}. The pantothenic acid requirement of chicks for growth appeared to be increased somewhat when the vitamin B_{12} supply was inadequate.

B. OF RATS

THOMAS H. JUKES and WILLIAM L. WILLIAMS

For many years, "synthetic" diets have been used for nutritional studies with rats. These diets have commonly contained a purified carbohydrate, such as starch, sucrose, glucose, or dextrin, together with casein as a source of protein. The casein of commerce is generally washed with dilute salt solution or treated alternately with alkali and acid to remove B-complex vitamins, or it may be extracted with a fat solvent to remove vitamin A. Yeast and wheat germ were often added in former years, but as the B-complex vitamins became available in synthetic form, it has been quite customary to replace the yeast and wheat germ with synthetic vitamin mixtures. It is possible to obtain reproduction in rats on diets of this type,[30] but breeding diets commonly used for the production of experimental rats are prepared with crude natural foods and are frequently supplemented with such materials as raw liver and fresh milk. This practice reflects the awareness that the nutritional requirements for reproduction in rats are more exacting than the needs for growth.

A series of investigations, particularly those of Coward, Mapson, Cary, Hartman, and their coworkers,[30-36] have led to the realization that casein contains an essential vitamin component which is not readily removed by washing with water and which is not present in yeast, cereal grains, or vegetable protein concentrates. This substance is now known to be vitamin

[29] H. Yacowitz, L. C. Norris, and G. F. Heuser, *J. Biol. Chem.* **192**, 141 (1951).
[30] T. H. Jukes, *Proc. Soc. Exptl. Biol. Med.* **45**, 625 (1940).
[31] K. H. Coward, K. M. Key, and B. G. E. Morgan, *Biochem. J.* **23**, 695 (1929).
[32] K. H. Coward, K. M. Key, F. J. Dyer, and B. G. E. Morgan, *Biochem. J.* **24**, 1952 (1930); **25**, 551 (1931).
[33] K. H. Coward, F. J. Dyer, R. A. Morton, and J. H. Gaddum, *Biochem. J.* **25**, 1102 (1931).
[34] L. W. Mapson, *Biochem. J.* **26**, 970 (1932).
[35] L. W. Mapson, *Biochem J.* **27**, 1061 (1933).
[36] A. M. Hartman, L. P. Dryden, and C. A. Cary, *J. Biol. Chem.* **140**, liv (1941).

B_{12}. It probably escaped major attention in nutritional studies with rats for many years because of its pronounced tendency to be carried over in the offspring from the well-supplemented diets which were fed to the mother rats and also because of the apparent tendency of the vitamin to adhere to casein which was thought to be vitamin-free. Both Coward and Cary were concerned with obtaining diets which were as low as possible in vitamin A. The approach to this problem involved extracting casein with hot alcohol to remove the last traces of vitamin A. This also removed vitamin B_{12}, so that a deficiency of this factor was developed in the offspring of mother rats which received the basal diet.

A deficiency of an unknown growth factor was shown by Coward and coworkers[31-33] to occur in rats on purified diets containing 8 % of dried yeast. The factor was present in some samples of casein but not in others and was termed the "light white casein factor." The factor was removed from casein by boiling with alcohol. Similar studies were reported by Mapson,[34] who used the name "physin" for a growth factor needed by rats and occurring in liver. Mapson found that female rats when fed liver transmitted the effect of "physin" to their offspring which had not received the supplement. The basal diet contained 10 % wheat germ and 8 % yeast, which are good sources of B-complex factors, except vitamin B_{12}. In a subsequent article[35] Mapson described the concentration of physin by extraction of a papain digest of fresh beef liver with 90 % alcohol and 90 % acetone. The final solution contained 380 mg. of solids from 100 g. of fresh liver and showed activity when fed in doses equivalent to 1 to 2 g. of original liver. Manganese was ineffective. Mapson[34] obtained negative results with Lilly's extract No. 343 and a liver extract ("B.D.H."), which presumably could be regarded as sources of the anti-pernicious anemia factor, and the trail was lost. It was picked up again by Cary, Hartman, and their coworkers, who carried out investigations during 1941 to 1949 which were described only briefly and usually in the form of abstracts. In 1941, they reported[36] that a vitamin A-free diet, similar to that of Sherman, plus cod liver oil in ample amounts was inadequate for optimum growth rate with weanling rats when the casein was extracted with hot alcohol. Growth was increased when the ration was supplemented with a liver extract. The deficiency could be demonstrated with weanling rats whose mothers' diets were changed at parturition to the basal diet.

Further studies were described in 1946.[15] Rats at weaning ordinarily contained a still unidentified factor (X) which affected their growth and development on a diet (A) adequate in all known nutrients. The young could be depleted of X by feeding their mothers a diet deficient in X. Casein prepared by centrifugation from milk was a good source of X, and commercial and various purified caseins contained different amounts of X; but, when 20 % or 40 % casein C, prepared by ten 6-hour extractions with hot alcohol,

was fed in diet A (containing 5% of yeast protein) to X-deficient rats, growth rates were 54% and 25% of normal, respectively. Coagulated egg albumin gave growth similar to that obtained with casein C, and with 60% of casein C the rats generally died within 2 weeks. On diet A with 25% of lactose and 20% of casein C growth was 36% of normal. When a few milligrams of certain commercial liver extracts were fed daily with diet A to sex-litter mates of the above rats, growth was normal or approximately normal except with 60% casein (85% normal). A few micrograms of a crude concentrate of X, fed separately, gave normal growth when tested with the 20% casein diet. The active factor in liver extracts was water-soluble, dialyzable, and precipitable with ammonium sulfate.

Further characteristics of the deficiency were described in a popular article by Cary and Hartman.[37] Increasing the protein in the basal ration, adding casein, egg albumin, or yeast, or feeding soybean meal or linseed oil meal in place of carbohydrates did not improve growth; in fact, the addition of soybean meal, linseed meal, or yeast actually depressed growth. In contrast, certain preparations of the growth-promoting nutrient concentrated from liver brought about optimum growth in daily doses of 20 γ and were active in daily doses of 2 γ. Furthermore, a supplement of liver extract enabled normal growth to take place in the rats fed diets containing additional amounts of soybean meal, linseed meal, and yeast. These important observations showed that these various high protein diets were not in themselves deleterious, for the unidentified factor enabled normal growth to take place when a high level of protein was fed.

Vitamin B_{12} was found to increase the rate of growth on all levels of protein under the same conditions as liver extract,[38] and a maximally effective dosage level of vitamin B_{12} produced practically the same effect as doses of liver extract which had been found to be maximally effective for male rats. The results are illustrated in Table XXII. Vitamin B_{12} also prevented the kidney hypertrophy which occurred on the basal diet.

The effect of all-vegetable diets on rats was studied by Zucker and Zucker,[39] who described a deficiency which was prevented by liver or crude casein but not by yeast. Cottonseed meal was used at high levels in the basal diets. In the year following, they reported that fish solubles and a concentrate prepared from cow manure by H. R. Bird's group were both active and suggested that this indicated a similarity between the deficiencies occurring in rats and chicks.[40] A number of interesting explorations of the natural distribution of the factor were made by the Zuckers, who found

[37] C. A. Cary and A. M. Hartman, *Yearbook of Agr. 1943–1947*, *U. S. Dept. Agr.* p. 779 (1947).

[38] A. M. Hartman, L. P. Dryden, and C. A. Cary, *Arch. Biochem.* **23**, 165 (1949).

[39] L. M. Zucker and T. F. Zucker, *Abstr. 111th Meeting, Am. Chem. Soc.* p. 45B (1947).

[40] T. F. Zucker and L. M. Zucker, *Vitamins and Hormones* **8**, 1 (1950).

that oysters were an even better source than liver. The richest natural source encountered was the eggs of the crustacean *Artemia salina* which had activity equivalent to 7.2 γ of vitamin B_{12} per gram of solids by assay with rats.

Emerson[22] studied the effects of vitamin B_{12} by the oral and subcutaneous

TABLE XXII

COMPARISON OF EFFECTS OF VITAMIN B_{12} AND LIVER EXTRACT ON GROWTH[38]

Casein level, %	Sets of littermate male rats	Duration of assay, days	Negative control average gain in weight, g.	Vitamin B_{12} (cryst.)		15-unit liver extract	
				Daily dose, γ	Average gain in weight, g.	Daily dose, ml.	Average gain in weight, g.
25	10	28	64	0.01	86	0.0025	120
25	3	14	35	2.5	78	0.05	81
25	5	14		5.0	78	0.10	85
45	4	14	22	2.8	74	0.10	72
65	2	14	4	5.0	53	0.10	55

The basal diet consisted of casein, extracted ten times with hot alcohol, dried yeast (10% of diet) dextrin, lactose, minerals, and vitamins without B_{12}).

TABLE XXIII

EFFECT OF VITAMIN B_{12} ON GROWTH OF MALE RATS RECEIVING A DIET CONTAINING 60% SOYBEAN MEAL AND 0.25% THYROID POWDER. AVERAGE WEIGHT AT START OF TEST PERIOD: 120 G.[22]

Supplement, daily	Gain in weight, 15 days
None	28
0.5 γ vitamin B_{12}, orally	79
0.5 γ vitamin B_{12}, subcutaneously	74
0.25 γ vitamin B_{12}, orally	72
0.25 γ vitamin B_{12}, subcutaneously	73
0.125 γ vitamin B_{12}, orally	65
0.125 γ vitamin B_{12}, subcutaneously	71
0.0625 γ vitamin B_{12}, orally	51
0.0625 γ vitamin B_{12}, subcutaneously	50

routes in rats fed a diet high in soybean meal and containing 0.25% of thyroid powder, U.S.P. Male rats averaging 40 g. in weight were placed at weaning on the basal diet for a depletion period of 28 days following which they were given the supplements shown in Table XXIII for 15 days. The results indicated that vitamin B_{12} was utilized equally well either orally or subcutaneously and that the requirement was almost satisfied by 0.125 γ daily for a rat averaging about 150 g. in weight during the course of the experiment. This places the requirement at about 0.8 γ per kilogram of body weight per day. This is markedly in excess of the requirement of a human

patient with pernicious anemia, whose needs appear to be satisfied by 1 to 2 γ injected daily. The requirements of chicks are similarly high (p. 511).

The oral administration of vitamin B_{12} at low dosage levels produces excellent responses in deficient rats. However, just as in the case of human subjects, the ingested vitamin was found not to be excreted in the urine of rats[41] unless it was given at such unusually high levels as 0.9 mg. per kilogram of body weight, in which case only a fraction of a per cent of the dose was present in the urine.

The relation between various alterations in dietary composition and vitamin B_{12} deficiency in the mouse was studied by Bosshardt and coworkers.[42] The addition of fat to vitamin B_{12}-deficient diets containing 20% or more protein eliminated fatalities and resulted in an increase in growth. The best growth was obtained when a fairly high level of fat was supplied in the diet together with vitamin B_{12}.

C. OF PIGS

THOMAS H. JUKES and WILLIAM L. WILLIAMS

The early approaches to the experimental production of vitamin B_{12} deficiency in pigs, just as with chicks, were made difficult by the presence of the vitamin in casein used in "purified" diets,[43] and again, as in the case of chicks, the use of crude all-vegetable diets often gave better experimental results than did the employment of "purified" diets. It soon became evident that pigs, like chicks, needed an animal protein factor.

A growth response of pigs on purified or all-vegetable diets to supplements of liver extract, fish meal, or fish solubles was noted by various investigators.[44-48] The diet used by McRoberts and Hogan contained acid-washed casein, sucrose, lard, corn starch, minerals, and vitamins and was homogenized into an "artificial milk" before feeding. Responses were obtained to extracts of yeast or liver.

Ferrin[47] stated that fish meal appeared to contain some dietary factors which were water-soluble, and he found that choline or methionine would not replace the growth-promoting effects of fish meal for pigs when added to a corn-soybean meal diet.

[41] B. F. Chow, L. Barrows, and C. Lang, *J. Nutrition* **42**, 405 (1950).
[42] D. K. Bosshardt, W. J. Paul, and R. H. Barnes, *J. Nutrition* **40**, 595 (1950).
[43] R. W. Colby and M. E. Ensminger, *J. Animal Sci.* **9**, 90 (1950).
[44] N. R. Ellis and J. H. Zeller, *J. Animal Sci.* **2**, 374 (1943).
[45] V. F. McRoberts and A. G. Hogan, *J. Nutrition* **28**, 165 (1944).
[46] B. W. Fairbanks, J. L. Krider, and D. V. Catron, *North Am. Veterinarian* **24**, 351 (1944).
[47] E. F. Ferrin, *J. Animal Sci.* **5**, 42 (1946).
[48] D. V. Catron and C. C. Culbertson, *Iowa Farm Sci.* **3**, 3 (1949).

Investigations by Hogan and Anderson[49] were made with a revision of Hogan's purified diet[45] to include pteroylglutamic acid and increased amounts of biotin. They concluded that the diet was slightly inadequate for growth and seriously inadequate for lactation. They found[50] that growth was accelerated by the administration of anti-pernicious anemia liver extract by intramuscular injection, but growth was not as rapid as when the purified diet was replaced by cow's milk fortified with sucrose and minerals. Vitamin B_{12} was used in an extension of the investigation,[51] and it was found that growth was greatly increased by injection of the vitamin. By a somewhat involved calculation, the authors estimated that the quantitative requirement of vitamin B_{12} when administered orally was 0.26 γ daily per kilogram of live weight or not over 15 γ per kilogram of food.

An anemia in a single pig which responded to the injection of commercial liver extract was described by Cartwright and coworkers.[52] Four other pigs receiving the same purified diet, except that commercial casein replaced the vitamin-free product, showed no signs of deficiency.

Purified diets were fed to young pigs by Neumann and coworkers.[53] The diets consisted of glucose, lard, minerals, vitamins, phthalylsulfathiazole, and either "vitamin-free" casein or "alpha" (soybean) protein plus methionine. This mixture was homogenized with water and fed to the pigs starting at 2 days of age. The effects of concentrated anti-pernicious anemia liver extract were studied, and the following results were obtained:

	Average daily gain during 8-week period, g.			
Experiment	Casein	Casein plus liver extract[a]	α-Protein	α-Protein plus liver extract[b]
1	232		210	327
2	257	357		

[a] 0.33 ml.
[b] 0.25 ml. of Reticulogen given by mouth daily.

Improvements in the physical appearance of the pigs were noted in the pigs receiving liver extract. Similar results were obtained in a subsequent experiment with liver extract,[54] and in addition the pigs responded to vitamin B_{12}, 2 γ injected daily, in the same manner as to liver extract. In contrast, the pigs on the basal α-protein diet grew very slowly and three died, all of which showed a loss of appetite, incoordination of the hind

[49] A. G. Hogan and G. C. Anderson, *J. Nutrition* **36**, 437 (1948).
[50] A. G. Hogan and G. C. Anderson, *J. Animal Sci.* **9**, 163 (1950).
[51] G. C. Anderson and A. G. Hogan, *J. Nutrition* **40**, 243 (1950).
[52] G. E. Cartwright, M. M. Wintrobe, and S. Humphreys, *J. Lab. Clin. Med.* **31**, 423 (1946).
[53] A. L. Neumann, J. L. Krider, and B. C. Johnson, *Proc. Soc. Exptl. Biol. Med.* **69**, 513 (1948).
[54] B. C. Johnson and A. L. Neumann, *J. Biol. Chem.* **178**, 1001 (1949).

quarters, unsteadiness, and a tendency to roll over on the side or back. Detailed pathological studies of the pigs described by Johnson and Neuman were reported by Thiersch[55] in collaboration with these authors. The deficient animals showed a number of departures from normal. These included increased size of the tongue and the liver, with vascularization and leucocytic infiltration in the papillary bodies of the squamous cell layers of the tongue. There were no apparent differences between groups with regard to the red blood cell series, but the deficient pigs showed an increase in the percentage of neutrophiles with a concomitant decrease in lymphocytes.

TABLE XXIV

MEAN BONE MARROWS BEFORE (A) AND 5 WEEKS AFTER (B) VITAMIN B_{12} INJECTION IN PIGS[55]

	Groups and Treatment				
	Natural diets	Basal diet		Basal + 2 γ vitamin B_{12} daily after 21st day	
		A	B	A	B
No. of pigs	5	2	1	3	1
% myeloid cells	76	57	20	40	71
% erythroid cells	23	40	78	47	13
% lymphoid cells	1	3	2	13	16
Differential of myeloids:					
Mature neutrophiles	36	64	40	54	73
Metamyelocytes	22	26	26	34	16
Myelocytes	29	7	30	10	8
Myeloblasts	1	1	0	1	0
Eosinophiles and basophiles	12	2	4	1	3
Differential of erythroids:					
Orthochromic normoblasts	86	94	50	78	74
Basophilic normoblasts	13	4	50	20	21
Macrocytic normoblasts	1	2		2	3

The bone marrow findings are illustrated in Table XXIV. Megaloblasts were not found, in contrast to their presence in the bone marrow of folic acid-deficient pigs in a report by Heinle and coworkers.[56] New lesions were not found in the spinal cord and sciatic nerves of pigs on the deficient diet in spite of their gross symptoms of posterior incoordination and unsteadiness of gait. Similar pathological findings were recorded with another series of pigs on various levels of added vitamin B_{12} ranging from 17 to 68 γ per kilogram of dry matter in the diet.[57]

[55] A. L. Neumann, B. C. Johnson, and J. B. Thiersch, *J. Nutrition* **40**, 403 (1950).

[56] R. W. Heinle, A. D. Welch, and J. A. Pritchard, *J. Lab. Clin. Med.* **33**, 1647 (1948).

[57] A. L. Neumann, J. B. Thiersch, J. L. Krider, M. F. James, and B. C. Johnson, *J. Animal Sci.* **9**, 83 (1950).

The effect of a "crude vitamin-B_{12} concentrate" on the growth of weanling pigs fed a corn-soybean diet was studied by Luecke and coworkers.[58] Six 6-week-old pigs were used in each group, and the pigs grew at about 1.4 times the rate of the control group during a period of 7 weeks; however, the authors noted that the supplementary preparation was impure so that it could not be definitely stated that its growth-promoting activity was due to its vitamin B_{12} content.

Further studies by the Illinois group[59] placed the vitamin B_{12} requirement of young pigs on the purified α-protein diet at about 20 γ per kilogram of dry diet or 0.6 γ per kilogram of body weight when injected daily; the latter figure was approximately half of the oral requirement.

In experiments with pigs on a corn-peanut meal basal diet, Cunha and associates[60] found that a vitamin B_{12} concentrate prepared from the streptomycin fermentation was ineffective in promoting growth while an animal protein factor supplement from the aureomycin fermentation produced a remarkable increase in growth. It was later shown by Jukes and coworkers[61] that vitamin B_{12} was ineffective under these conditions and that small amounts of aureomycin were responsible for the growth produced by the animal protein factor supplement.

The requirement of male pigs on wire floors for vitamin B_{12} on a corn-soybean meal diet was studied by Richardson and coworkers[62] in the presence and absence of 40 mg. of supplementary antibiotics per pound of diet. The following results were obtained:

Level of added vitamin B_{12}, γ per pound diet	Gain in pounds per day from weaning to 75 lb.	
	Without antibiotics	With antibiotics
0	1.19	0.91
5	1.22	1.54
10	1.25	1.58
20	1.29	1.53

Pigs receiving the basal diet and antibiotics without additional vitamin B_{12} showed an extremely rough hair coat, dermatitis, an exudate around the eyes, nervousness, and irritability. The authors concluded that the supplementary vitamin B_{12} requirement of weanling pigs was 5 γ or less per pound of corn-soybean meal diet containing antibiotics.

[58] R. W. Luecke, W. N. McMillen, F. Thorp, Jr., and J. R. Boniece, *Science* **110**, 139 (1949).

[59] R. O. Nesheim, J. L. Krider, and B. C. Johnson, *Arch. Biochem.* **27**, 240 (1950).

[60] T. J. Cunha, J. E. Burnside, D. M. Buschman, R. S. Glasscock, A. M. Pearson and A. L. Shealy, *Arch. Biochem.* **23**, 324 (1949).

[61] T. H. Jukes, E. L. R. Stokstad, R. R. Taylor, T. J. Cunha, H. M. Edwards, and G. B. Meadows, *Arch. Biochem.* **26**, 324 (1950).

[62] D. Richardson, D. V. Catron, L. A. Underkofler, H. M. Maddock, and W. C. Friedland, *J. Nutrition* **44**, 371 (1951).

D. OF RUMINANTS

THOMAS H. JUKES and WILLIAM L. WILLIAMS

In many parts of the world it has long been recognized that ruminants are subject to a characteristic nutritional deficiency disease which in 1935[63, 64] was shown to be due to a lack of cobalt. A lack of adequate quantities of available cobalt in the soil leads to a low content of this element in forage and other crops which is reflected in a malady occurring in sheep and cattle which subsist on these crops. The disease is variously and colloquially known in different localities as salt sickness, pining, coast disease, and bush sickness.

The observation that cobalt was effective by mouth but not by injection[65] led to the suggestion that cobalt produced the response by acting upon some of the microorganisms in the rumen.[66] Non-ruminants are not known to be subject to the disease;[67] indeed, horses and rabbits can grow and reproduce on the forage crops grown in the deficient areas without the appearance of dietary disturbance. It now appears that ruminants suffering from the disease respond to injections of vitamin B_{12}.

The deficiency in sheep has been described as being characterized by loss of appetite, listlessness, anemia, lethargy, and weakness terminating in death. The pathological examination at autopsy has shown nothing specific, although hemosiderosis of the liver, spleen, and pancreas has often been noted.[68] Filmer[69] reported that the feeding of whole liver would cure the disease in cattle and sheep although it was later concluded that liver contained insufficient cobalt to account for its beneficial effect.[70] Presumably cobalt in the form of vitamin B_{12} might be quantitatively more effective than elemental cobalt in the oral treatment of the disease in ruminants, thus accounting for these results with the feeding of liver.

The first experiments with vitamin B_{12} were inconclusive, apparently as a result of underdosage,[71-73] and led to wrong interpretations. The responses obtained in pernicious anemia with as little as 1 γ of vitamin B_{12} daily may have led to underestimation of the vitamin B_{12} requirement of rumi-

[63] H. R. Marston and E. W. L. Lines, *J. Council Sci. Ind. Research* **8**, 11 (1935).
[64] E. J. Underwood and J. F. Filmer, *Australian Vet. J.* **11**, 84 (1935).
[65] C. J. Martin, cited by McCance and Widdowson, 1944 (ref. 66).
[66] R. A. McCance and E. M. Widdowson, *Ann. Rev. Biochem.* **13**, 315 (1944).
[67] J. F. Thompson and G. H. Ellis, *J. Nutrition* **34**, 121 (1947).
[68] H. R. Marston, *Ann. Rev. Biochem.* **8**, 570 (1939).
[69] J. F. Filmer, *Australian Vet. J.* **9**, 163 (1933).
[70] J. F. Filmer and E. J. Underwood, *Australian Vet. J.* **13**, 57 (1937).
[71] H. R. Marston and H. J. Lee, *Nature* **164**, 529 (1949).
[72] D. E. Becker, S. E. Smith, and J. K. Loosli, *Science* **110**, 71 (1949).
[73] W. H. Hale, A. L. Pope, P. H. Phillips, and G. J. Bohstedt, *J. Animal Sci.* **8**, 621 (1949).

nants, which actually does not appear to differ greatly from that of chicks and rats when computed on the basis of body weight. It was found by Becker and Smith[74] that the injection of 15 U.S.P. units of concentrated liver extract was effective for sheep, and Johnson[75] noted that calves on purified diets containing inorganic cobalt showed a deficiency which was alleviated by vitamin B_{12}. Hale and coworkers[76] suggested that the earlier doses of vitamin B_{12} may have been too small. They obtained partial responses in two cobalt-deficient lambs which received, respectively, 100 γ of vitamin B_{12} injected daily and 200 γ by mouth daily for 4 weeks. Even better responses were given by other cobalt-deficient lambs which were given a mixture of B vitamins not including B_{12}. Smith[77] and Marston[78] also concluded that the amounts of vitamin B_{12} given in their earlier studies were insufficient. It was reported[77] that cobalt deficiency was induced in lambs by feeding them a diet of low-cobalt hay, shelled corn, and milk powder containing approximately 0.03 part of cobalt per million. The typical signs of deficiency were quickly alleviated when the lambs were injected with 150 γ or more of vitamin B_{12} over a 2-week period. In a second experiment, orally administered cobalt salts were compared with injections of vitamin B_{12} and B_{12b}. No significant differences were obtained among the three treatments, and the lambs all responded well, gaining an average of 0.35 lb. daily.

The reason for the observation that non-ruminants on cobalt-deficient forage crops do not develop vitamin B_{12} deficiency remains unexplained. Perhaps the non-ruminants have a lower requirement for vitamin B_{12}, or their intestinal synthesis or absorption of vitamin B_{12} is more efficient than that of ruminants. The finding that B-complex vitamins other than vitamin B_{12} alleviated cobalt deficiency in lambs[73] is also unexplained.

E. OF HUMAN BEINGS

FRANK H. BETHELL

Vitamin B_{12} is probably the most biologically active of the known vitamins in terms of the amount required by the organism. Although B_{12} is an essential nutrient, it appears that normal nutrition and blood cell production can be maintained in almost complete absence of a dietary supply of the material. In such cases, where the diet has been lacking in foods of

[74] D. E. Becker and S. E. Smith, *J. Animal Sci.* **8**, 615 (1949).

[75] B. C. Johnson, Gordon Research Conferences, American Association for the Advancement of Science, 1950.

[76] W. H. Hale, A. L. Pope, P. H. Phillips, and G. J. Bohstedt, *J. Animal Sci.* **9**, 484 (1950).

[77] S. E. Smith and B. A. Koch, *J. Animal Sci.* **10**, 1062 (1951).

[78] H. R. Marston, Personal communication, 1951.

animal origin, the nutritional needs must, presumably, be met by intestinal bacterial synthesis of the vitamin. The presence of relatively large amounts of B_{12}, or substances with B_{12} activity, has been demonstrated in the feces, even in patients with pernicious anemia,[79] but the quantity present in the contents of the small intestine is small.[80] How much of the B_{12} produced within the alimentary tract may be in an absorbable form or present at sites favorable for absorption is unknown.

Persons whose diets contain milk, eggs, and meat probably ingest a more than adequate supply of B_{12}. Thus, cow's milk was found to contain 6.6 γ per liter,[81] and the values for beef, pork, ham, mutton, and veal ranged between 3 and 10 γ per 100 g.[82]

Since B_{12} in a daily parenteral dose of 1 γ induces complete remission in pernicious anemia patients,[83, 84] the inference appears justified that the normal daily requirement may be met by the absorption of 1 γ from the alimentary tract. It appears probable, however, that only a fraction of the B_{12} naturally present in food is absorbed even under normal digestive conditions.

[79] R. H. Girdwood, *Trans. Roy. Soc. Trop. Med. Hyg.* **42**, 65 (1948).
[80] R. H. Girdwood, *Edinburgh Med. J.* **58**, 309 (1951).
[81] G. M. Briggs and R. J. Lillie, *Federation Proc.* **5**, 124 (1946).
[82] V. J. Lewis, U. D. Register, H. T. Thompson, and C. A. Elvehjem, *Proc. Exptl. Biol. Med.* **72**, 479 (1949).
[83] B. E. Hall and D. C. Campbell, *Proc. Staff Meetings Mayo Clinic* **23**, 584 (1948).
[84] F. H. Bethell, M. C. Meyers, and R. B. Neligh, *J. Lab. Clin. Med.* **33**, 1477 (1948).

Chapter 4

BIOTIN

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I. Nomenclature and Formula

ROBERT S. HARRIS

Accepted name: Biotin[1]

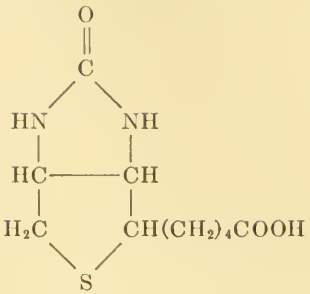
Obsolete names: Bios II[2] Factor X[3]
Bios IIB[4] Coenzyme R[5]
Vitamin H[6] Anti-egg white injury factor[7]
Factor W[8] Skin factor[9, 10]

Empirical formula: $C_{10}H_{16}O_3N_2S$

Chemical name: *cis*-Hexahydro-2-oxo-1H-thieno(3,4)-imidazole-4-valeric acid (or 2′-keto-3,4-imidazolido-2-tetrahydrothiophene-*n*-valeric acid)

[1] F. Kögl, *Ber. deut. chem. ges.* **68**, 16 (1935).
[2] G. H. W. Lucas, *J. Phys. Chem.* **28**, 1180 (1924).
[3] M. A. Boas, *Biochem. J.* **21**, 712 (1927).
[4] W. Lash Miller, *Trans. Roy Soc. Canada* **III, 30**, 99 (1936).
[5] F. E. Allison, S. R. Hoover, and D. Burk, *Science* **78**, 217 (1933).
[6] P. György, *Z. ärztl. Fortbildung* **28**, 377, 417 (1931).
[7] J. G. Lease and H. T. Parsons, *Biochem J.* **28**, 2109 (1934).
[8] C. A. Elvehjem, C. J. Koehn, and J. J. Oleson, *J. Biol. Chem.* **115**, 707 (1936).
[9] W. Marshall, *J. Invest. Dermatol.* **2**, 205 (1939).
[10] W. Marshall, *Med. World* **57**, 101 (1939).

Structure:



Biotin

II. Chemistry

PAUL GYÖRGY

A. ISOLATION

The identification of biotin with the curative factor (vitamin H) for egg white injury[1] helped to merge the originally independent studies dealing with a microbiological yeast factor (biotin) and a vitamin-like substance (vitamin H). Furthermore, it has been suggested[1] that biotin and vitamin H are also identical with coenzyme R, a growth and respiration factor for many strains of legume nodule bacteria.[2] The identity of biotin and coenzyme R had previously been indicated by other investigators.[3, 4]

Biotin as growth factor for yeast has been isolated in the form of a crystalline methyl ester from egg yolk by Kögl and Tönnis.[5]

Vitamin H was first recognized as the curative factor for egg white injury. The toxicity of egg white as a food constituent was first observed by Bateman[6] and confirmed, at least under special conditions, by several other investigators in the past.[7-12] Rats fed a ration that was well bal-

[1] P. György, D. B. Melville, D. Burk, and V. du Vigneaud, *Science* **91**, 243 (1940).
[2] F. E. Allison, S. R. Hoover and D. Burk, *Science*, **78**, 217 (1933).
[3] R. Nilsson, G. Bjalfoe, and D. Burström, *Naturwissenschaften* **27**, 389 (1939).
[4] P. M. West and P. W. Wilson, *Science* **89**, 607 (1939).
[5] F. Kögl and B. Tönnis, *Hoppe-Seyler's Z. physiol. Chem.* **242**, 43 (1936).
[6] W. G. Bateman, *J. Biol. Chem.* **26**, 263 (1916).
[7] M. A. Boas, *Biochem. J.* **21**, 712 (1927).
[8] E. Friedberger and S. Seidenberg, *Deut. med. Wochschr.* **53**, 1507 (1927).
[9] F. Stenquist, *Deut. med. Wochschr.* **54**, 1920 (1928).
[10] P. György, *Z. ärztl. Forbild.* **28**, 377, 417 (1931).
[11] H. T. Parsons and E. Kelly, *J. Biol. Chem.* **100**, 645 (1933).
[12] H. T. Parsons and E. Kelly, *Am. J. Physiol.* **104**, 150 (1933).

anced except for a large proportion of egg white as the sole source of protein developed peculiar and impressive skin changes. These were accompanied by progressive emaciation and led finally to a fatal outcome. Diets rich in raw or slightly cooked fresh whole egg, egg white, or commercial Chinese dried egg white have been found equally detrimental. The egg white was found to be deprived to its toxic properties by continued and intensive treatment with heat, as well as by digestion with pepsin or incubation with hydrochloric acid, probably owing to hydrolysis.[11] An organic protective substance, present in different foodstuffs, was found likewise to be able to neutralize the toxic effect of egg white when the protective substance was given as a supplement to the otherwise disease-producing diet rich in egg white. This substance has been called the "protective factor X" by Boas,[7] vitamin H by György,[10] or "the factor protective against egg-white injury" by Lease and Parsons.[13]

Isolation of the active substance was first achieved even before identification of biotin with vitamin H (the anti-egg white injury factor) from egg yolk.[5] The yolks of 1000 fresh eggs were treated with acetone, and the filtrate after concentration was precipitated with four volumes of alcohol. The active principle was found in the precipitate, which was dissolved in water and the impurities removed by precipitation with lead acetate. The filtrate was freed from lead and treated with phosphotungstic acid. The precipitate, containing the active factor, was decomposed with baryta, and the solution was shaken with charcoal. The adsorbate was first washed with 50% alcohol, followed by elution with 60% acetone containing 2.5% of ammonia, leading to an active fraction. A second precipitation with phosphotungstic acid and decomposition with baryta gave an active fraction soluble in alcohol. Treatment with mercuric chloride removed impurities. After esterification with methanolic hydrogen chloride further impurities were removed by precipitation with picrolonic acid and then with rufianic acid. Finally, a very potent preparation was obtained by decomposition of the reineckate. From a second preparation, using dried egg yolk as starting material, the methyl ester was obtained in crystalline form by high vacuum distillation and crystallization from a mixture of chloroform and light petroleum. The substance had a m.p. of 146 to 147° and was active on *Saccharomyces* in a dilution of 1 in 10,000,000,000.

Later, Kögl and Pons[14] succeeded by the use of molecular distillation to purify further the crude product and even to increase the yield. The biotin methyl ester was crystallized from mesityl oxide which gave a purer product, m.p. 161 to 165°.

Before the identity of biotin with vitamin H (the anti-egg white injury

[13] J. G. Lease and H. T. Parsons, *Biochem. J.* **28**, 2109 (1934).
[14] F. Kögl and L. Pons, *Hoppe-Seyler's Z. physiol. Chem.* **269**, 61 (1941).

factor) was recognized, isolation of vitamin H was attempted from its most potent sources, i.e., liver and yeast, and no attention was given to egg yolk as starting material. It has been shown that vitamin H as it occurs in yeast and liver could not be dissolved in water or in any other solvent, at least not in sufficient quantity to be effective in the prevention of egg white injury in rats.[15] Autolysis of yeast in the presence of toluene led to liberation of vitamin H in a water-soluble form. Chloroform, on the other hand, inhibited reversibly this apparently fermentative reaction. On the basis of this observation the conclusion was drawn that biotin (vitamin H) is present in food products such as yeast and liver, mainly in bound form, and may be liberated, at least from yeast, by fermentative action.

In view of the fact that liver was found to be a more potent source of the curative factor of egg white injury than is yeast,[15] the question arose as to whether or not vitamin H (biotin) could be separated also by autolysis of liver tissue from the water-insoluble complex in which it occurs. Several attempts to do this under varying experimental conditions and again using animal bioassays for vitamin H failed.[16] In contrast, digestion with pepsin and even more with papain,[16-18] but not with trypsin, or autoclaving at high pressure,[16] with or without the addition of acid, have proved satisfactory procedures for the liberation of vitamin H (biotin) from a dry liver powder.

In these studies only animal assays were used. After the identification of biotin with vitamin H, microbiological test methods made the study of bound and free biotin easier and more accessible to a quantitative and better controlled analysis. With the more sensitive microbiological assay Snell *et al.*[19] found that autolysis increased the amount of biotin that could be extracted from liver, whereas in animal assays using egg white injury as test object such release of biotin from bound form was not demonstrable.[16] Acid or enzymatic hydrolysis gave maximum yields of biotin from a variety of natural materials, not only in microbiological tests[20-22] but also in animal assays.[16]

Before the identification of vitamin H with biotin the first isolation of vitamin H was attempted from the alcohol-insoluble fraction of beef liver

[15] P. György, *J. Biol. Chem.* **131**, 733 (1939).
[16] P. György, R. Kuhn, and E. Lederer, *J. Biol. Chem.* **131**, 745 (1939).
[17] J. G. Lease and H. T. Parsons, *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.* **105**, 1 (1934).
[18] J. G. Lease, *Z. Vitaminforsch.* **5**, 110 (1936).
[19] E. E. Snell, R. E. Eakin, and R. J. Williams, *J. Am. Chem. Soc.* **62**, 175 (1940).
[20] V. H. Cheldelin, M. A. Eppwright, E. E. Snell, and B. M. Guirard, *Univ. Texas Publ.* **4237**, 15 (1942).
[21] R. C. Thompson, R. E. Eakin, and R. J. Williams, *Science* **94**, 589 (1941).
[22] J. O. Lampen, G. P. Bahler, and W. H. Peterson, *J. Nutrition* **23**, 11 (1942).

in which the proportion of free vitamin H was increased by high-pressure hydrolysis.[16] After precipitation with alcohol and acetone the filtrate containing the active principle was treated with phosphotungstic acid, and the active precipitate decomposed with barium hydroxide.[16] This crude material, containing approximately 0.1 % biotin (vitamin H) was subjected to esterification conditions with methanol and HCl, and the ethyl acetate-soluble material from this procedure was dissolved in chloroform and passed through a column of activated aluminum oxide.[23] The column was then eluted in succession with chloroform, acetone, and then repeatedly with a mixture of 90 % acetone and 10 % methanol. By this procedure a fortyfold concentration of the active principle was accomplished. A repetition of the same chromatographic procedure achieved a further threefold concentration. The active material from this second elution was dissolved in chloroform, and the solution was extracted with dilute hydrochloric acid. After second esterification of this acid extract, crystals of the methyl ester of biotin were obtained after concentration of the ethyl acetate solution of the esterified active substance. The crystals were purified by two crystallizations from methanol-ether mixtures, and then by sublimation in a high vacuum (10^{-5} mm.). The sublimate was crystallized from methanol and ether in long, fine needles, m.p. 166 to 167° (uncorrected). The ester was subjected to several recrystallizations and sublimations *in vacuo* without changing its biological activity and melting point.[23, 24] Expressed in terms of vitamin H units, the crystalline ester had a potency of 27,000 vitamin H units per milligram. The same order of activity was also found for a crystalline biotin preparation obtained by Kögl,[5] as further proof for the identity of biotin and vitamin H.[24]

With a modification of the procedure used for the isolation of biotin from liver, Melville *et al.*[25] have succeeded in obtaining pure biotin from a crude milk concentrate which is a more readily available natural source of biotin. After the usual esterification procedure the biotin ester was adsorbed on Decalso from chloroform solution and eluted with a 5 % methanol-acetone mixture. The eluate was passed through a column of commercial activated alumina. The column was treated with a 10 % methanol-acetone mixture from which, after removal of the solvent, the crystalline methyl ester was obtained. Further purification consisted in washing with ethyl acetate, followed by sublimation *in vacuo* and crystallization from a meth-

[23] V. du Vigneaud, K. Hofmann, D. B. Melville, and P. György, *J. Biol. Chem.* **140**, 643 (1941).

[24] P. György, C. S. Rose, K. Hofmann, D. B. Melville, and V. du Vigneaud, *Science* **92**, 609 (1940).

[25] D. B. Melville, K. Hofmann, E. Hague, and V. du Vigneaud, *J. Biol. Chem.* **142**, 615 (1942).

anol-(peroxide free) ether mixture, again with a melting point of 166 to 167°.

B. CHEMICAL AND PHYSICAL PROPERTIES

The biotin methyl ester is soluble in methanol, ethanol, acetone, and chloroform, sparingly soluble in ethyl acetate, and almost insoluble in water and ether. It is optically active; $[\alpha]_D^{22} = +57°$ for a 1 % solution in chloroform.

The correct empirical formula, $C_{11}H_{18}O_3N_2S$, for biotin methyl ester

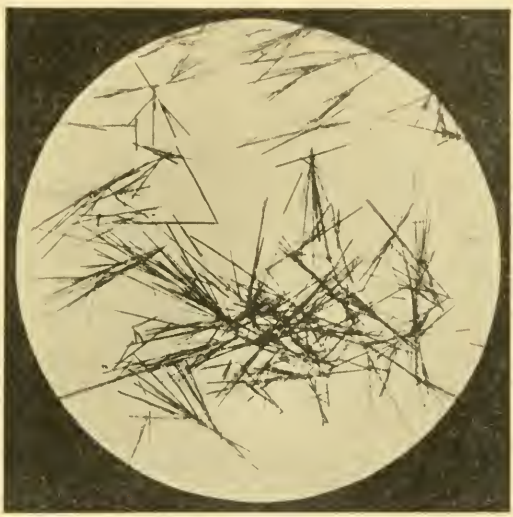


FIG. 1. Biotin (free acid) crystallized from water (magnification 100 ×). From D. B. Melville. *Vitamins and Hormones* **2**, 40 (1944).

was reported in 1937 by Kögl,[26] and confirmed by du Vigneaud *et al.*[23] Spectroscopic studies revealed no specific bands for biotin methyl ester in the range from 2200 to 6000 A.[14, 27]

The biotin methyl ester is readily saponified with dilute alkali at room temperature. After acidification with dilute hydrochloric acid and concentration free biotin is obtained in crystalline form from the saponification mixture.[27] After recrystallization from water the long fine needles (Fig. 1) of free biotin melt with some decomposition at 230 to 232° (uncorrected). Free biotin is soluble in dilute alkali and hot water, sparingly soluble in dilute acid and cold water, and practically insoluble in organic solvents.

[26] F. Kögl, *Naturwissenschaften* **25**, 465 (1937).

[27] V. du Vigneaud, K. Hofmann, D. B. Melville, and J. R. Rachele, *J. Biol. Chem.* **140**, 763 (1941).

A 0.3% solution of the compound in 0.1 *N* NaOH showed an optical activity of $[\alpha]_D^{22} = +92°$.[27]

C. CONSTITUTION

1 Functional Groups

The easy saponification of biotin methyl ester indicated the loss of one methylene group. Re-esterification of the free acid could be accomplished by

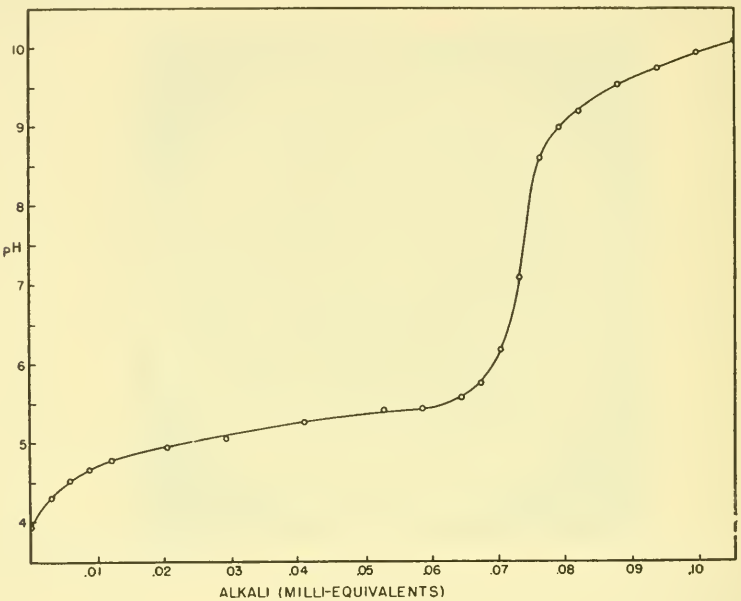


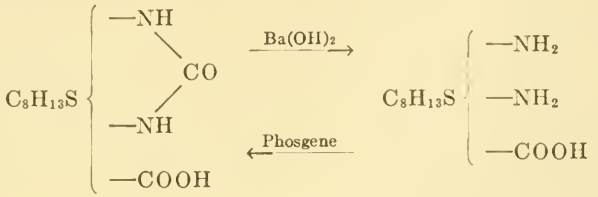
Fig. 2. Electrometric titration of 17.99 mg. of biotin with NaOH. From D. B. Melville, *Vitamins and Hormones* **2**, 42 (1944).

the use of diazomethane: the methyl ester produced in this manner was identical with that isolated from esterified liver fractions. Biotin, $C_{10}H_{16}O_3N_2S$, in contrast to the methyl ester, did not liberate volatile iodides when treated with HI and consequently does not contain $—OCH_3$, NCH_3, or SCH_3 groups. Electrometric titration of biotin yielded a curve (Fig. 2) resembling that of a monocarboxylic acid.[27] Biotin crystallized from strongly acid solutions in form of the free acid. This fact, together with the shape of the titration curve for biotin, indicates that the basic group of groups present in the molecule were very weak. No inactivation occurred,[28] and no color was produced[29] when the substance was treated with ninhydrin. Further, no nitrogen was formed when biotin was inactivated by the Van Slyke

[28] K. Hofmann, D. B. Melville, and V. du Vigneaud, *J. Biol. Chem.* **141**, 207 (1941).

amino nitrogen procedure.[29] These results show that biotin contains neither an amino group nor basic ring nitrogen atoms.

Through drastic hydrolysis of biotin with hydrochloric acid[14] or barium hydroxide[28] an optically active diaminocarboxylic acid, $C_9H_{18}O_2N_2S$, was obtained which was found to contain two primary amino groups by the Van Slyke nitrous acid method. Various derivatives of this compound were prepared, such as dibenzoate, diacetate, sulfate, dihydrochloride, and dipicrolonate. The formation of the strongly basic diaminocarboxylic acid, $C_9H_{18}O_2N_2S$, from the weakly basic biotin, $C_{10}H_{16}O_3N_2S$, with the replacement of one CO group by two hydrogen atoms, pointed to a cyclic urea structure. This assumption was well supported by the resynthesis of biotin from the diaminocarboxylic acid with phosgene.[30]

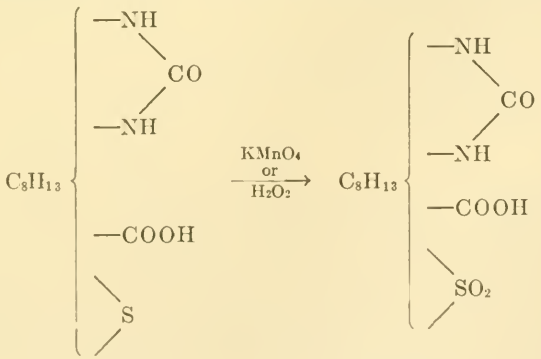


Biotin Diaminocarboxylic acid

The methyl ester of the resynthesized product was identical with the original natural biotin methyl ester. Their yeast growth activities were also equal.

2. The Nature of the Sulfur in Biotin

The sulfur of biotin appeared to be stable. No H_2S was liberated when biotin was treated with zinc dust and HCl, and after treatment with bromine no inorganic sulfate could be detected. The nitroprusside test for



Biotin Biotin sulfone

[29] G. B. Brown and V. du Vigneaud, *J. Biol. Chem.* **141**, 85 (1941).

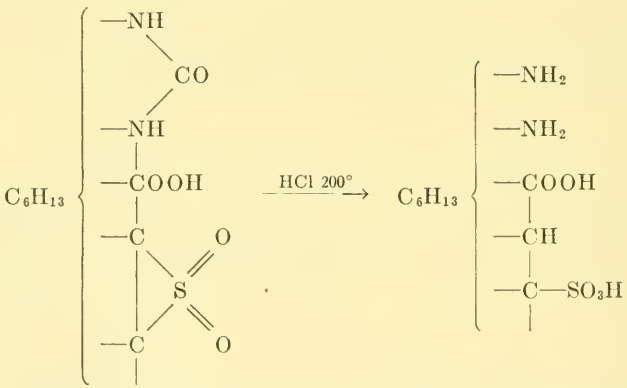
[30] D. B. Melville, K. Hofmann, and V. du Vigneaud, *J. Biol. Chem.* **145**, 101 (1942).

sulfhydryl groups was negative. Oxidation with cold potassium permanganate or with H_2O_2 (in glacial acetic acid) revealed an uptake of two atoms of oxygen, with the formation of biotin sulfone[28, 31] with the empirical formula of $C_{10}H_{16}O_5N_2S$.

The formation of a sulfone established the thio ether nature of the sulfur atom, and it was, therefore, concluded that biotin is a monocarboxylic acid containing a cyclic urea structure with the sulfur atom in thio ether linkage.

In the light of the empirical formula of biotin, in the absence of an ethylenic linkage, and with the knowledge of the functional groups, it could be calculated that biotin must contain a bicyclic ring system with the sulfur atom as part of one of the rings.

Kögl and de Man[31] hydrolyzed biotin sulfone at 200° with concentrated hydrochloric acid and obtained a new compound to which they assigned the structure of an aliphatic 9-carbon diaminocarboxysulfonic acid. They assumed that during this forced hydrolysis the urea ring is opened, resulting in the formation of the sulfone of the diaminocarboxylic acid, and in addition one of the carbon-sulfur linkages is ruptured to yield the open-chain sulfonic acid derivative as indicated below.

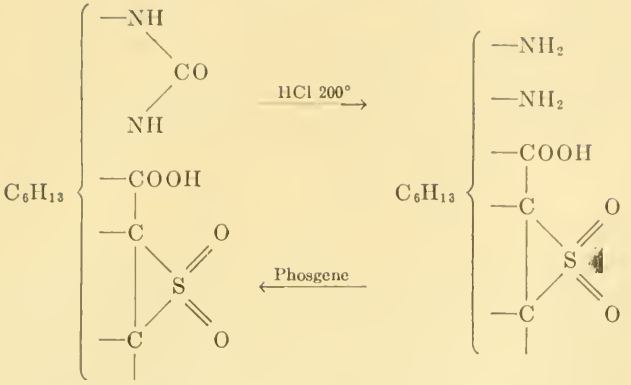


Biotin sulfone

9-Carbon diaminocarboxysulfonic acid

Melville, Hofmann, and du Vigneaud[30] have been unable to demonstrate a rupture of sulfur-containing ring after treatment of biotin sulfone with concentrated hydrochloric acid at 200°. In their experiments such treatment resulted only in the formation of the sulfone of the diaminocarboxylic acid from which biotin sulfone was resynthesized in high yield by treatment with phosgene. Thus, drastic hydrolysis of biotin sulfone does not affect the sulfur part of the molecule.

[31] F. Kögl and T. J. de Man, *Hoppe-Seyler's Z. physiol. Chem.* **269**, 81 (1941).



Biotin sulfone

Sulfone of the diaminocarboxylic acid

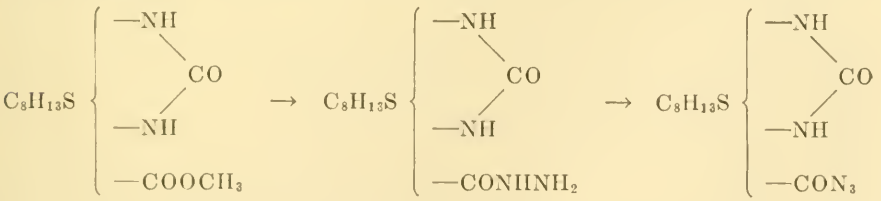
3. The Side Chain in the Biotin Molecule

Valuable information on the structure of biotin was obtained during studies of the oxidative breakdown of the diaminocarboxylic acid with either alkaline permanganate or with concentrated nitric acid. Such oxidation resulted in the formation of adipic acid, $C_6H_{10}O_4$.[32, 33]

$$HOOC—CH_2—CH_2—CH_2—CH_2—COOH$$

Adipic acid

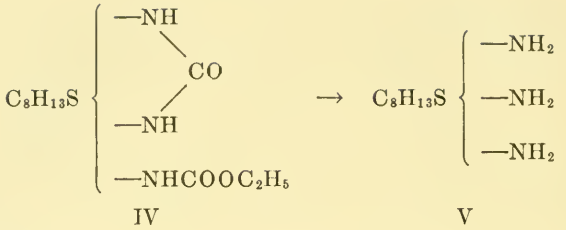
The isolation of this straight-chain dicarboxylic acid containing six of the carbon atoms of the diaminocarboxylic acid permitted two possible interpretations. The adipic acid might have arisen from an aliphatic side chain in biotin, or from a 6-carbon ring cleaved by oxidation. In the former case the original carboxyl group of biotin would appear as one of the carboxyl groups of adipic acid; in the latter case neither of the carboxyl groups of the adipic acid would be the original carboxyl group in biotin. These two possibilities were put to test by means of a Curtius degradation of biotin methyl ester.[33] In this way the carboxyl group of the diaminocarboxylic acid was replaced by an amino group, as indicated by the following formulas.



I II III

[32] K. Hofmann, D. B. Melville, and V. du Vigneaud, *J. Am. Chem. Soc.* **63**, 3237 (1941).

[33] K. Hofmann, D. B. Melville, and V. du Vigneaud, *J. Biol. Chem.* **144**, 513 (1942).

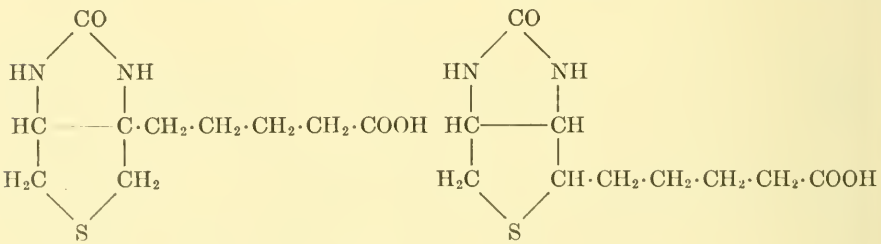


IV V

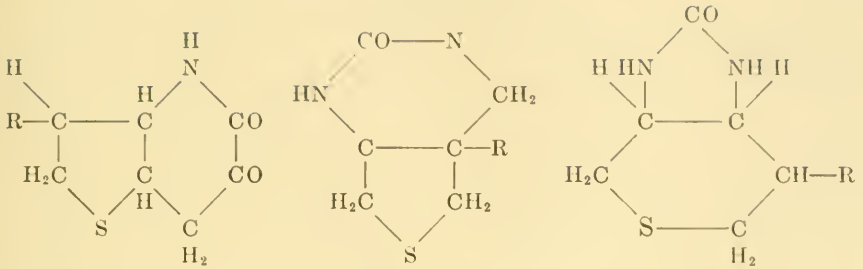
Biotin methyl ester (I) was converted into biotin hydrazide (II) by treatment with hydrazine hydrate, and the corresponding azide (III) was prepared from the hydrazide with the calculated amount of nitrous acid without affecting the urea part of the molecule. When the azide was boiled with ethanol, the corresponding ethyl urethane (IV) resulted. The urethane was hydrolyzed with barium hydroxide to the triamine (V), thus simultaneously opening the urea ring.

The triamine was subjected to the same oxidation procedures which had been employed for the oxidation of the diaminocarboxylic, but no adipic acid could be identified among the oxidation products. Thus, one of the two carboxyl groups of the adipic acid must have been originally present in the diaminocarboxylic acid, and it became highly probable that the adipic acid formed by oxidation of the diaminocarboxylic acid arose, not from a cyclic structure but from an aliphatic side chain —CH_2—CH_2—CH_2—CH_2—COOH attached to a ring carbon in the biotin molecule.

The marked stability of the diaminocarboxylic acid toward hydrolytic agents rendered unlikely structures in which the sulfur atom and an amino group were attached to the same carbon atom. Such structures would be unstable toward alkali and acid. This fact, together with the oxidative formation of adipic acid, favored the assumption that biotin might contain a tetrahydrothiophene nucleus with a *n*-valeric acid chain attached in the β or α position, as depicted in formulas VI and VII. However, the possibility could not have definitely been ruled out that the adipic acid might have been formed from the decarboxylation of a malonic or α-substituted



VI VII



VIII IX X

R = —$CH_2 \cdot CH_2 \cdot CH_2 \cdot COOH$

β-keto acid arising during the oxidation, with the consequence of three additional structural possibilities, VIII, IX, and X.[34]

X-ray diffraction study gave no unequivocal answer in favor of any of the above five possibilities.[35]

4. The Size of the Urea Ring

In three of the discussed structural formulas the urea rings were five-membered, whereas the remaining two were trimethylene derivatives.

The size of the urea ring was established by condensation of the diaminocarboxylic acid with phenanthrenequinone.[36] A crystalline quinoxaline derivative was thus obtained, $C_{23}H_{20}O_2N_2S$, m.p. 202 to 204°. Although the formation of quinoxaline derivatives by the condensation of 1,2-diamines with orthoquinones is well established, no examples of the condensation of 1,3-diamines with phenanthrenequinone could be found in the literature.

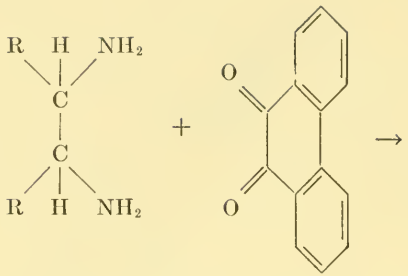
Two different compounds, dihydroquinoxalines or quinoxalines, may be obtained through the condensation of non-aromatic 1,2-diamines with phenanthrenequinone, according to the reaction on following page

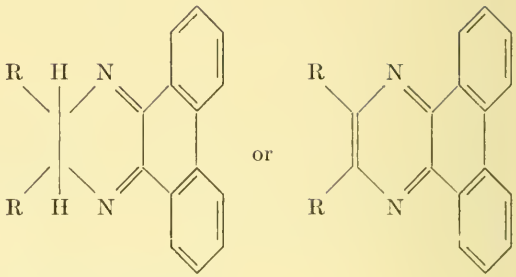
The analytical composition as well as certain color reactions of the condensation product derived from biotin appeared to favor the quinoxaline structure rather than that of a dihydroquinoxaline. The ultraviolet absorption spectrum of the condensation product was compared with the spectra of the quinoxaline and dihydroquinoxaline derivatives obtained

[34] V. du Vigneaud, K. Hofmann, and D. B. Melville, *J. Am. Chem. Soc.* **64**, 188 (1942).

[35] I. Fankuchen, *J. Am. Chem. Soc.* **64**, 1742 (1942).

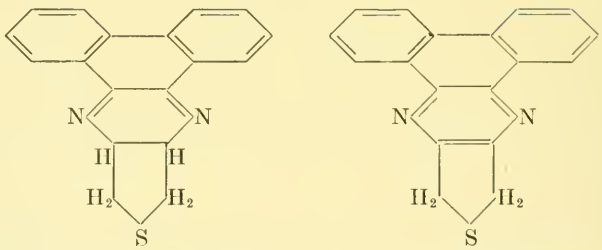
[36] K. Hofmann, G. W. Kilmer, D. B. Melville, V. du Vigneaud, and H. H. Darby, *J. Biol. Chem.* **145**, 503 (1942).





Dihydroquinoxaline Quinoxaline

by condensing 3,4-diaminotetrahydrothiophene[37] with phenanthrenequinone.



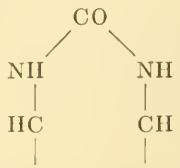
3,4-Diaminothiophene (dihydroquinoxaline) 3,4-Diaminothiophene (quinoxaline)

As shown in Fig. 3, the absorption curve of the derivative obtained from biotin was very similar to that of the quinoxaline derivative from 3,4-diaminothiophene and was distinctly different from that pertaining to the dihydroquinoxaline. This finding strongly indicated that the condensation product of the diaminocarboxylic acid with phenanthrenequinone is a dibenzoquinoxaline derivative. Thus, the 1,2-diamine structure of the diaminocarboxylic acid was firmly established, providing proof for the

[37] G. W. Kilmer, M. D. Armstrong, G. B. Brown, and V. du Vigneaud, *J. Biol. Chem.* **145**, 495 (1942).

FIG. 3. Ultraviolet absorption spectra of the condensation product of phenanthrenequinone with (1) the diaminocarboxylic acid from biotin; (2) 3,4-diaminotetrahydrothiophene, oxidized form; (3) 3,4-diaminotetrahydrothiophene, reduced form. From D. B. Melville, *Vitamins and Hormones* **2**, 55 (1944).

presence of a five-membered urea ring in biotin, in which the carbon atoms bearing the amino groups must carry hydrogen atoms.



Of the possible biotin structures (pp. 536, 537) only formulas VII and X may comply with these conclusions; the other three are eliminated from further consideration.

5. Desthiobiotin

Further fairly conclusive evidence for the correct structure of biotin was furnished by the study of "desthiobiotin," the compound corresponding to biotin with the sulfur atom removed and replaced by two hydrogen atoms. Bougault *et al.*[38] have shown that treatment of disulfide with Raney

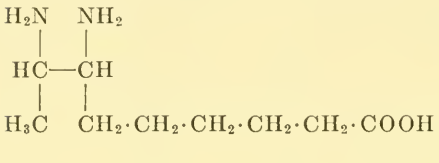
[38] J. Bougault, E. Cattelain, and P. Chabrier. *Bull. Soc. chim. France* **7**, 781 (1940).

nickel in alcoholic solution is followed by cleavage of the molecule according to the following formula.

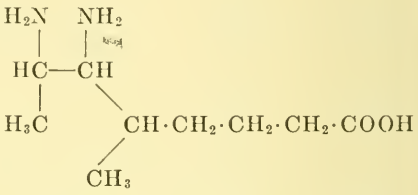
$$R—S—S—R^1 \xrightarrow{\text{Raney nickel}} R—SH + HS—R^1$$

Similar treatment of organic sulfides results with good yield in cleaved products from which the sulfur is removed. In the case of biotin methyl ester the end product is desthiobiotin methyl ester, $C_{11}H_{20}O_3N_2$, which by hydrolysis with either $Ba(OH)_2$ at 140° or concentrated HCl at 200° gives the desthiodiaminocarboxylic acid, $C_9H_{20}O_2N_2$.[39]

If formula VII (p. 536) represents the correct structure of biotin, desulfurization and hydrolysis of the urea ring should end in ζ,η-diaminopelargonic acid, and in δ-methyl-ϵ,ζ-diaminocaprylic acid if biotin possesses the structure shown by formula X.



ζ,η-Diaminopelargonic acid

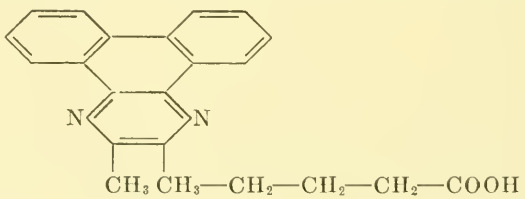


δ-Methyl-ϵ,ζ-diaminocaprylic acid

Carbon methyl determination and oxidative cleavage of the desthiodiaminocarboxylic acid favored the structure corresponding to ζ,η-diaminopelargonic acid with one carbon methyl group and pimelic acid as oxidative cleavage product.

Condensation of the desthiodiaminocarboxylic acid with phenanthrenequinone resulted in the formation of the quinoxaline, $C_{23}H_{22}O_2N_2$.[38] In contradistinction to dihydroquinoxaline the compound gave a red color with sulfuric acid and in its ultraviolet absorption spectrum it was very similar to that of the quinoxaline derivative of 3,4-diaminotetrahydrothiophene.

If the quinoxaline is formed from the diaminopelargonic acid, the resulting product should be represented by the following formula.

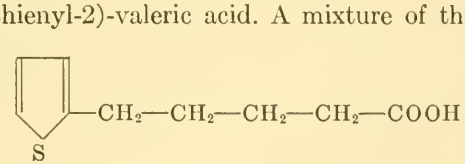


[39] V. du Vigneaud, D. B. Melville, K. Folkers, D. E. Wolf, R. Mozingo, J. C. Keresztesy, and S. A. Harris, *J. Biol. Chem.* **146**, 475 (1942).

This compound possesses no centers of asymmetry, since in its formation the asymmetry of the two carbon atoms bearing the amino groups is destroyed. In contrast, the other possible desthiodiaminocarboxylic acid should still possess an asymmetric carbon atom. In fact the quinoxaline prepared from desthiobiotin proved to be optically inactive and in its chemical and physical characteristics identical with the quinoxaline prepared from the corresponding synthetic diamonopelargonic acid. It is obvious from these data that of the two possible structures (VII and X), only structure VII agreed with the fact observed in the study of desthiobiotin (p. 536).

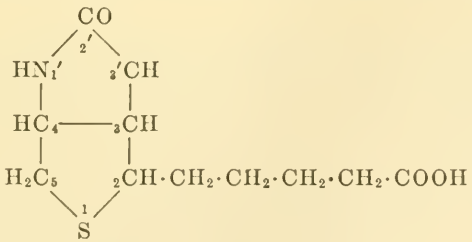
6. The Structure of Biotin

Final proof for the correct structure of biotin was obtained by exhaustive methylation studies on the diaminocarboxylic acid from biotin.[40] Treatment with dimethylsulfate and alkali, followed by decomposition with strong hydrochloric acid, resulted in the formation of a sulfur-containing acid which was isolated in crystalline form (m.p. 40 to 41°) and identified as δ-(thienyl-2)-valeric acid. A mixture of this compound with



the corresponding synthetic δ-(thienyl-2)-valeric acid showed no depression of the melting point and gave identical color reactions. As shown in Fig. 4, in which the wavelength is plotted against the specific extinction coefficient, both compounds showed identical ultraviolet absorption curves, with a maximum absorption at 234 mμ.

In view of all these data, obtained through different analytical methods, the correct formula of biotin must be represented by the following structure.



Biotin

2'-Keto-3,4-imidazolido-2-tetrahydrothiophene-*n*-valeric acid

[40] D. B. Melville, A. W. Moyer, K. Hofmann, V. du Vigneaud, *J. Biol. Chem.* **146**, 487 (1942).

7. α- AND β-BIOTINS

The chemical and physical characteristics of biotin isolated from liver apply equally well for biotin obtained from milk and two other sources.[41] In contrast, Kögl and his coworkers[42-45] have put forward the claim that biotin exists in natural products in two chemically closely related forms with comparable biological properties. Biotin originally obtained by Kögl from egg yolk[5, 46] differed in melting point and specific rotation from biotin

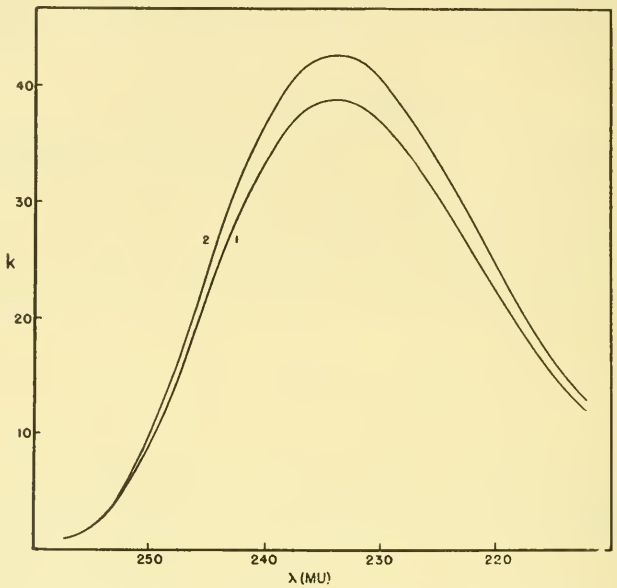
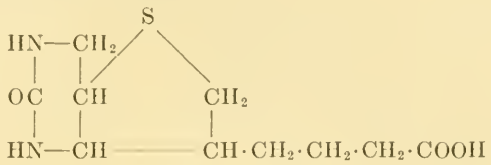


FIG. 4. Ultraviolet absorption spectra of (1) the compound isolated from biotin; (2) synthetic δ-(α-thienyl)valeric acid, in 95% alcohol. The wavelength is plotted against the specific extinction. From D. B. Melville, *Vitamins and Hormones* **2**, 62 (1944).

isolated by Kögl from liver.[43] Kögl further stated that a mixture of the free acids and of their methyl esters from liver and egg yolk showed a depression of melting point. X-ray analyses confirmed the non-identity of the two substances. The compounds from egg yolk and liver were

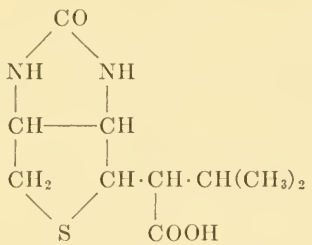
[41] D. B. Melville, *Vitamins and Hormones* **2**, 66 (1944).
[42] F. Kögl and W. A. J. Borg, *Hoppe-Seyler's Z. physiol. Chem.* **281**, 65 (1944).
[43] F. Kögl and E. J. ten Ham, *Hoppe-Seyler's Z. physiol. Chem.* **279**, 140 (1943).
[44] F. Kögl, H. Erxleben, and J. H. Verbeek, *Hoppe-Seyler's Z. physiol. Chem.* **276**, 63 (1942).
[45] F. Kögl, J. H. Verbeek, H. Erxleben, and W. A. J. Borg, *Hoppe-Seyler's Z. physiol. Chem.* **279**, 121 (1943).
[46] F. Kögl, *Ber.* **68**, 16 (1935).

designated by Kögl α-biotin and β-biotin, respectively. β-Biotin should be identical with the biotin isolated by the American workers. However, there are discrepancies in the optical activity reported for the β-biotin methyl ester by the American workers and by Kögl (+57° and +39° in chloroform, respectively). For β-biotin from liver Kögl and ten Ham[43] suggested the following structure.



This formula has been ruled out by du Vigneaud and his coworkers as a possible structure for biotin isolated from liver.

Without going into details of the chemical analysis employed by Kögl and his coworkers,[42-45] the following final formula was proposed by them for α-biotin isolated from egg yolk.



The proof presented by Kögl in favor of this structure is not convincing. In the absence of confirming evidence and especially lacking support by successful synthesis, the question whether α-biotin exists as a separate and well-established isomer of biotin must be left open and should be viewed with justified skepticism. In this connection it is of interest that a supposedly pure α-biotin preparation obtained from Kögl proved[47] to possess 90 to 96 % of the activity of pure β-biotin—even of synthetic β-biotin (with correction for the inactivity of (−)-biotin—for *Lactobacillus casei* 7469, *L. pentosus* 124-2, *Saccharomyces cerevisiae* Y-30, *Clostridium acetobutylicum* S-9, and *Neurospora crassa* (1-A wild). It is exceedingly unlikely that for a wide variety of organisms two chemically non-identical forms of biotin should have essentially identical biological activity. In the following discussion only biotin with the structure (p. 541) elucidated by du Vigneaud and his coworkers will be considered and the distinction between α and β forms will not be maintained.

[47] K. K. Krueger and W. H. Peterson, *J. Biol. Chem.* **173**, 497 (1948).

D. SYNTHESIS OF BIOTIN

The conclusions regarding the structure of biotin as they arose from the excellent investigations of du Vigneaud and his coworkers received complete confirmation by the synthesis of biotin first accomplished by Harris and his colleagues in the Merck laboratory.[48-52] In this synthesis cysteine (I) was condensed with chloroacetic acid as starting materials. After benzoylation and esterification this condensation product (II) was treated with sodium methoxide. The resulting compound (V) has undergone decarboxylation after treatment with hydrochloric acid in an aqueous acetic acid solution (VI). Compound VI contains the tetrahydrothiophene ring, one amino group, and one potential amino group in the ketone.

The methyl γ-formylbutyrate used to introduce the valeric acid side chain was prepared from glutaric acid as starting material. Glutaric acid anhydride (VII) was treated with methyl alcohol to give glutaric acid methyl ester (VIII), which was in turn converted to γ-carbomethoxybutyryl chloride (IX), and finally on treatment with a Rosenmund catalyst and hydrogen was converted to the aldehyde (X).

The condensation of the ketotetrahydrothiophene (VI) with the aldehydo acid (X) was achieved (XI) with the use of piperidine and acetic acid as catalyst. The unsaturated ketone gave after treatment with hydroxylamine in pyridine solution the unsaturated oxime XII. This compound contains the complete carbon skeleton of biotin with two nitrogen atoms and the sulfur atom in the proper position. The reduction of the oximino group and of the double bond to a completely saturated compound was accomplished in two steps. First, treatment of compound XII with zinc dust and acetic acid in the presence of acetic anhydride resulted in two compounds (XIII and XIV) which differed only by the position of the double bonds. In the second step, hydrogenation of these compounds, using a palladium catalyst and hydrogen gas, yielded completely saturated isomeric compounds (XV). Saponification with NaOH resulted in compound XVI. Both compounds XV and XVI were converted, following the procedure of du Vigneaud and his coworkers,[28] after treatment with barium hydroxide, to a diaminocarboxylic acid XVII. As the corresponding natural product, this synthetic diaminocarboxylic acid yielded upon treatment with

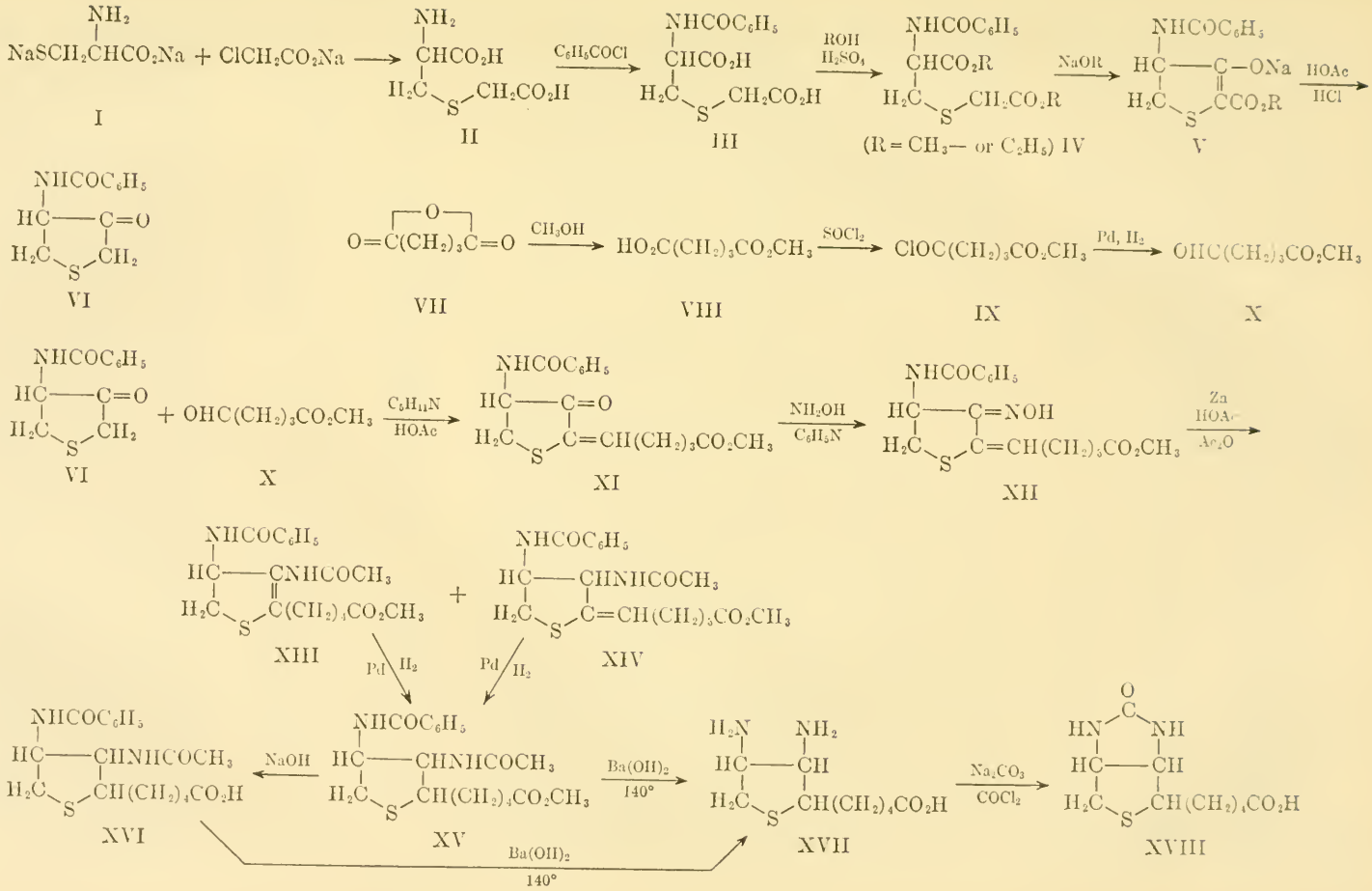
[48] S. A. Harris, D. E. Wolf, R. Mozingo, and K. Folkers, *Science* **97**, 447 (1943).

[49] S. A. Harris, D. E. Wolf, R. Mozingo, R. C. Anderson, G. E. Arth, N. R. Easton, D. Heyl, A. N. Wilson, and K. Folkers, *J. Am. Chem. Soc.* **66**, 1756 (1944).

[50] S. A. Harris, N. R. Easton, D. Heyl, A. N. Wilson, and K. Folkers, *J. Am. Chem. Soc.* **66**, 1757 (1944).

[51] S. A. Harris, R. Mozingo, D. E. Wolf, A. N. Wilson, G. E. Arth, and K. Folkers, *J. Am. Chem. Soc.* **66**, 1800 (1944).

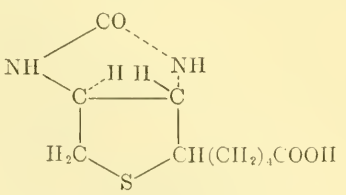
[52] S. A. Harris, D. E. Wolf, R. Mozingo, G. E. Arth, R. C. Anderson, N. R. Easton, and K. Folkers, *J. Am. Chem. Soc.* **67**, 2096 (1945).


NaSCH2CHCO2Na + ClCH2CO2Na
I
II
III
(R = CH3— or C2H5) IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI
XVII
XVIII
C6H5COCl
ROH
H2SO4
NaOR
HOAc
HCl
CH3OH
SOCl2
Pd, H2
C5H11N
HOAc
NH2OH
C6H5N
Zn
HOAc
Ac2O
Pd
H2
NaOH
Ba(OH)2
140°
Na2CO3
COCl2

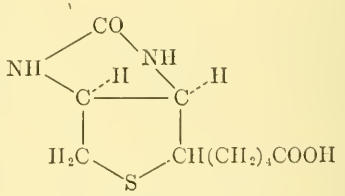
sodium carbonate and phosgene the cyclic urea compound XVIII, having the structure of the natural biotin.

The synthesis of biotin was complicated by the fact that it contained three asymmetric carbon atoms. Thus, there should exist four diastereoisomers or eight optically active modifications corresponding to the structure of biotin. All the racemic diastereoisomers, *dl*-biotin,[49-52] *dl*-epibiotin,[53] *dl*-allobiotin,[49-52] and *dl*-epiallobiotin,[51, 52] have been synthesized. *dl*-Biotin (m.p. 232°) and *dl*-allobiotin (m.p. 232°) originate from compound XIII; *dl*-epibiotin (m.p. 190 to 191°) and *dl*-epiallobiotin (fuses above 195°) from compound XIV.

The structure of these various racemic forms was successfully elucidated by their conversion into the corresponding desthio derivatives, using reduction with Raney nickel as first employed on natural biotin.[39] In desthiobiotin the asymmetry of the original carbon atom 2 in the biotin molecule is destroyed, and in consequence the resulting compound allows only two instead of four racemic forms. *dl*-Biotin and *dl*-epibiotin yielded the same *dl*-desthiobiotin, while the allo forms furnished among themselves identical *dl*-allodesthiobiotin. Thus, biotin and epibiotin differ by being epimeric at carbon 2 where the side chain is attached. The same conclusion applies to the allo forms. Compared with the epimeric biotins the allobiotins show easier conversion to diamino compounds and are in turn less easily reconverted by the action of phosgene into the original than are epimeric biotins. The instability of the urea group in *dl*-allobiotin and *dl*-epiallobiotin indicates a strained transfusion of the two rings[54] with a *trans* configuration of the nitrogens in the urea group (XIX). In contrast, *dl*-biotin and *dl*-epibiotin must have a *cis* ring structure (XX).



Allobiotin, epiallobiotin
XIX



Biotin, epibiotin
XX

dl-Biotin contained only one-half the biological activity of natural biotin. Its resolution into its two components met first with difficulties since it did not form crystallizable salts with ordinary alkaloids. The resolution was successfully accomplished by combining the racemic synthetic product

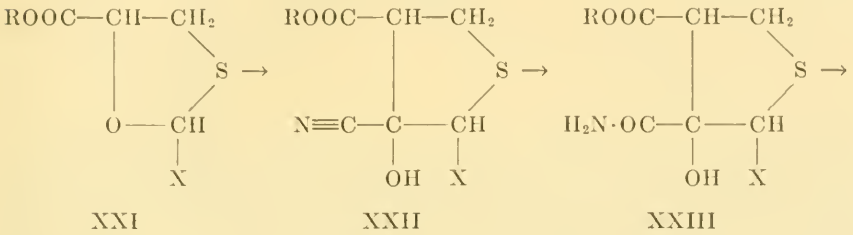
[53] B. R. Baker, W. L. McEwen, and W. N. Kinley, *J. Org. Chem.* **12**, 322 (1947).

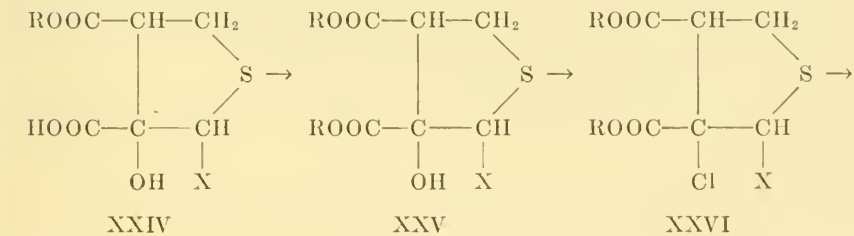
[54] S. A. Harris, R. Mozingo, D. E. Wolf, A. N. Wilson, and K. Folkers, *J. Am. Chem. Soc.* **67**, 2102 (1945).

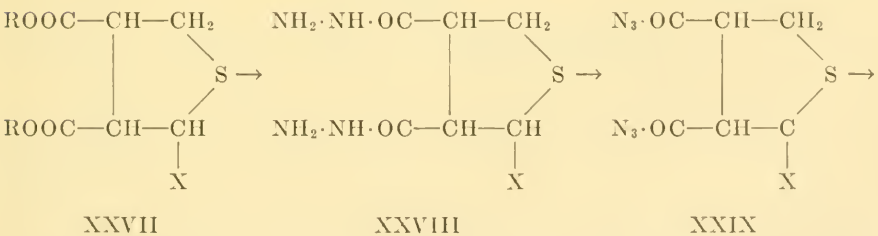
with optically active mandelic acid as an ester and fractionally crystallizing to separate the two compounds. The best method of preparing *d*-biotin was by means of the *l*-arginine salt.

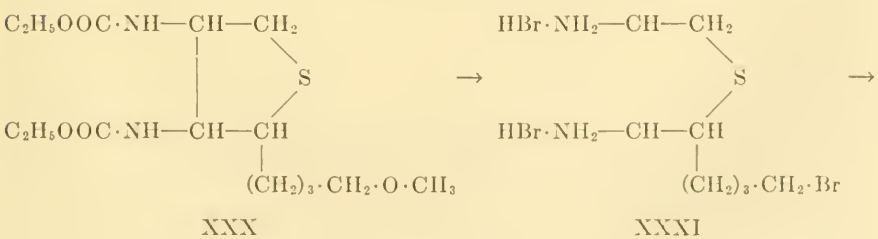
A very different synthesis of biotin was achieved by Grüssner *et al.*[55] from 2-δ-methoxybutyl-3-keto-4-carbethoxythiophane as shown in the reactions XXI–XXXIII.

R = $-CH_3$ or $-C_2H_5$ X = $-CH_2-CH_2-CH_2-CH_2\cdot O\cdot CH_3$

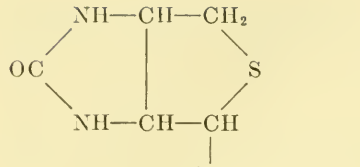




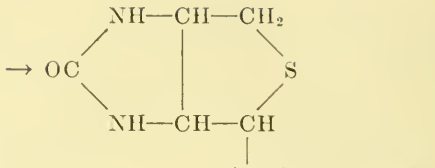




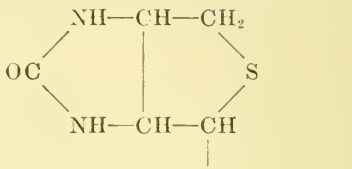
[55] A. Grüssner, J. P. Bourquin, and O. Schnider, *Helv. Chim. Acta* **28**, 517 (1945).



XXXII



XXXIII



Biotin

It was first claimed[55] that in this synthesis three racemic isomers were obtained, one of which was found to be identical with *dl*-biotin. Inasmuch as the two other compounds did not yield either *dl*-desthiobiotin or *dl*-allodesthiobiotin, it was later concluded[56] that they must be structurally, and not only stereochemically, different from biotin.

Another method of synthesis was described by Baker and his associates.[53, 57-62] In one method the following series of steps was employed (XXXIV–XLII).

$$C_2H_5OOC\cdot(CH_2)_4\cdot COCl \xrightarrow{H_2} C_2H_5OOC\cdot(CH_2)_4\cdot CHO \xrightarrow{\text{malonic acid}}$$

XXXIV XXXV

$$C_2H_5OOC\cdot(CH_2)_4\cdot CH{:}CH\cdot COOH \longrightarrow CH_3OOC\cdot(CH_2)_4\cdot CH{:}CH\cdot COOCH_3$$

XXXVI XXXVII

$$\xrightarrow{HS\cdot CH_2\cdot COOCH_3} CH_3\cdot OOC\cdot(CH_2)_4\cdot \underset{\displaystyle S\cdot CH_2\cdot COOCH_3}{\underset{|}{CH}}\cdot CH_2\cdot COOCH_3 \xrightarrow[+\ \text{toluene}]{NaOCH_3}$$

XXXVIII

[56] A. Grüssner, J. P. Bourquin, and O. Schnider, *Helv. Chim. Acta* **29**, 770 (1946).

[57] B. R. Baker, M. V. Querry, S. R. Safir, and S. Bernstein, *J. Org. Chem.* **12**, 138 (1947).

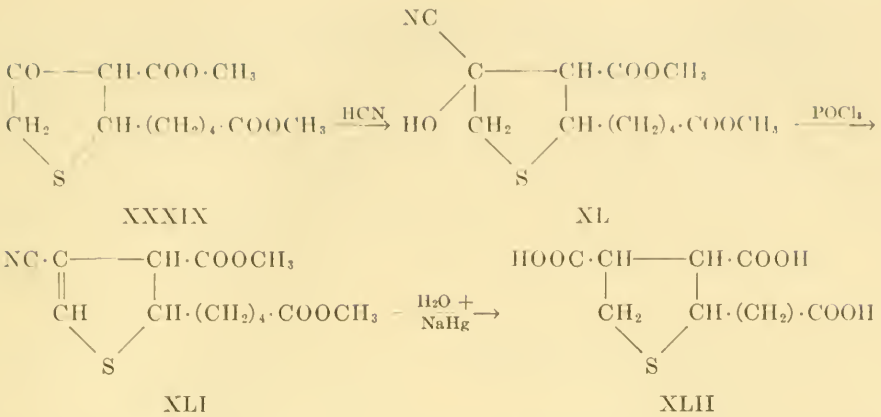
[58] G. B. Brown, B. R. Baker, S. Bernstein, and S. R. Safir, *J. Org. Chem.* **12**, 155 (1947).

[59] G. B. Brown, M. D. Armstrong, A. W. Moyer, W. P. Onslow, B. R. Baker, M. V. Querry, S. Bernstein, and S. R. Safir, *J. Org. Chem.* **12**, 160 (1947).

[60] B. R. Baker, M. V. Querry, S. Bernstein, S. R. Safir, and Y. SubbaRow, *J. Org. Chem.* **12**, 167 (1947).

[61] B. R. Baker, M. V. Querry, S. R. Safir, W. L. McEwen, S. Bernstein, *J. Org. Chem.* **12**, 174 (1947).

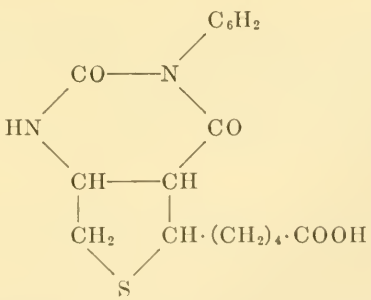
[62] B. R. Baker, M. V. Querry, W. L. McEwen, S. Bernstein, S. R. Safir, L. Dorfman, and Y. SubbaRow, *J. Org. Chem.* **12**, 186 (1947).



XXXIX XL

XLI XLII

This tricarboxylic acid (XLII) was converted into 2-(δ-carboxybutyl)-3,4-diaminothiophane by the selective degradation of the two nuclear carboxyl groups without affecting the side-chain carboxyl group. On treatment with phosgene this diaminocarboxylic yielded *dl*-epiallobiotin. Biotin with the *cis* configuration was prepared by a rather different series of reactions from a common intermediate, a *cis*-uracil with the structure:



A different type of synthesis is used for the industrial preparation of biotin.[63]

E. SYNTHESIS OF BIOTIN ANALOGS

1. Desthiobiotin

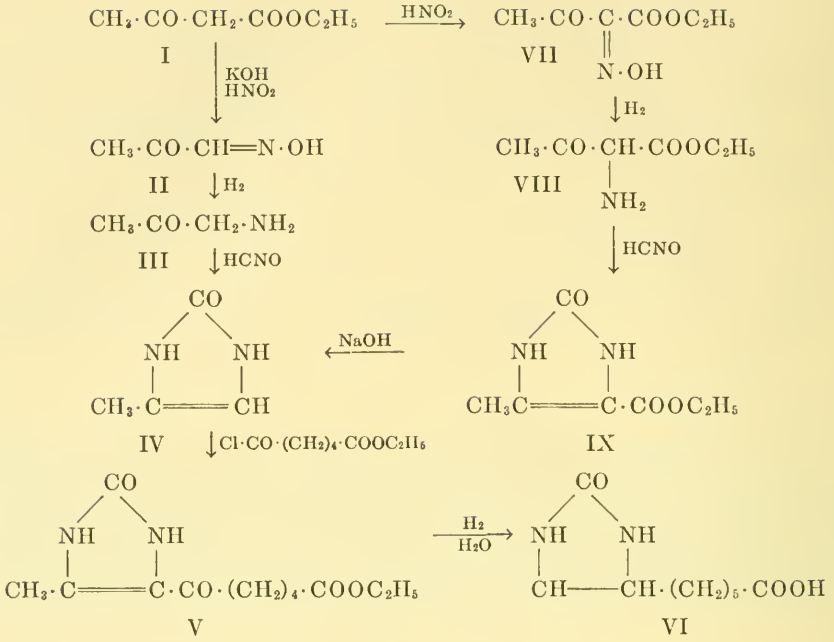
The synthesis of desthiobiotin was accomplished in several ways.

Melville[64] prepared it by the action of phosgene in alkaline solution on its degradation product ζ,η-diaminopelargonic acid. Duschinsky and Dolan[65] used the following series of reactions (I–IX).

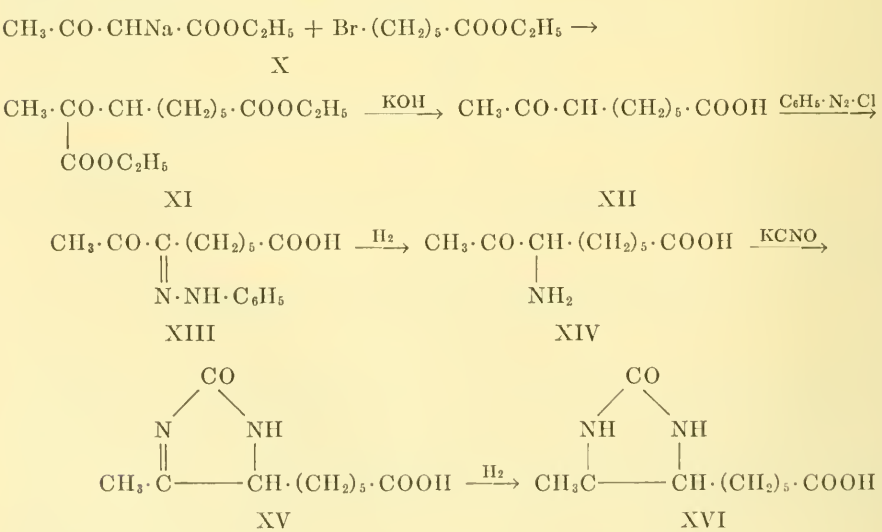
[63] M. W. Goldberg and L. H. Sternbach, U. S. Pats. 2,489,232–2,489,238, November 22, 1949.

[64] D. B. Melville, *J. Am. Chem. Soc.* **66**, 1422 (1944).

[65] R. Duschinsky and L. A. Dolan, *J. Am. Chem. Soc.* **67**, 2079 (1945).



Another method was used by Wood and du Vigneaud,[66] and again another, although similar, by Bourquin *et al.*[67] In the latter synthesis the following reactions were employed (X–XVI).

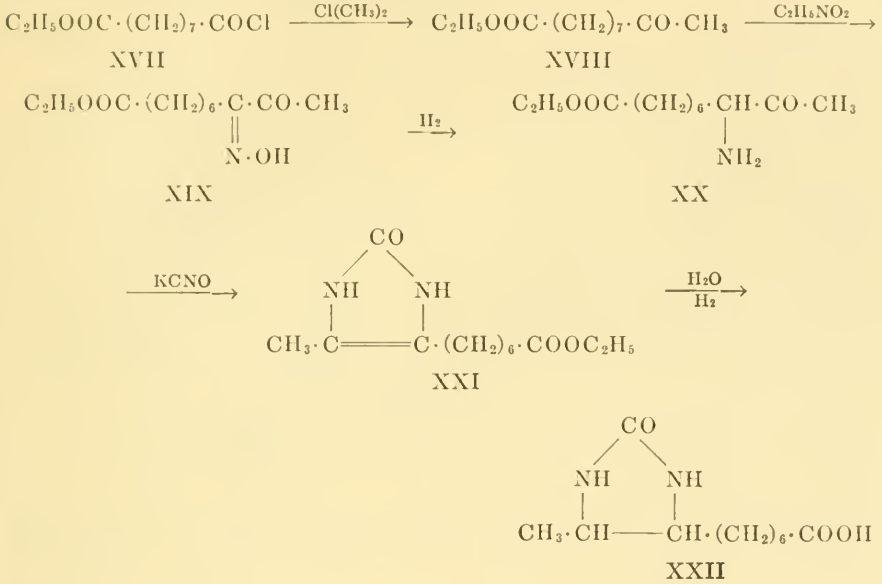


[66] J. L. Wood and V. du Vigneaud, *J. Am. Chem. Soc.* **67**, 210 (1945).

[67] J. P. Bourquin, O. Schnider, and A. Grüssner, *Helv. Chim. Acta* **28**, 528 (1945).

dl-Nordesthiobiotin was synthesized in a similar manner. A very similar method for preparing desthiobiotin was used by Swain,[68] except that the amino group was introduced by chlorination with sulfuryl chloride, followed by amination with potassium phthalimide.

A further method for the synthesis of desthiobiotin, also applicable for the synthesis of its higher homolog, homodesthiobiotin, was devised by McKennis and du Vigneaud[69] (XVII–XXII).

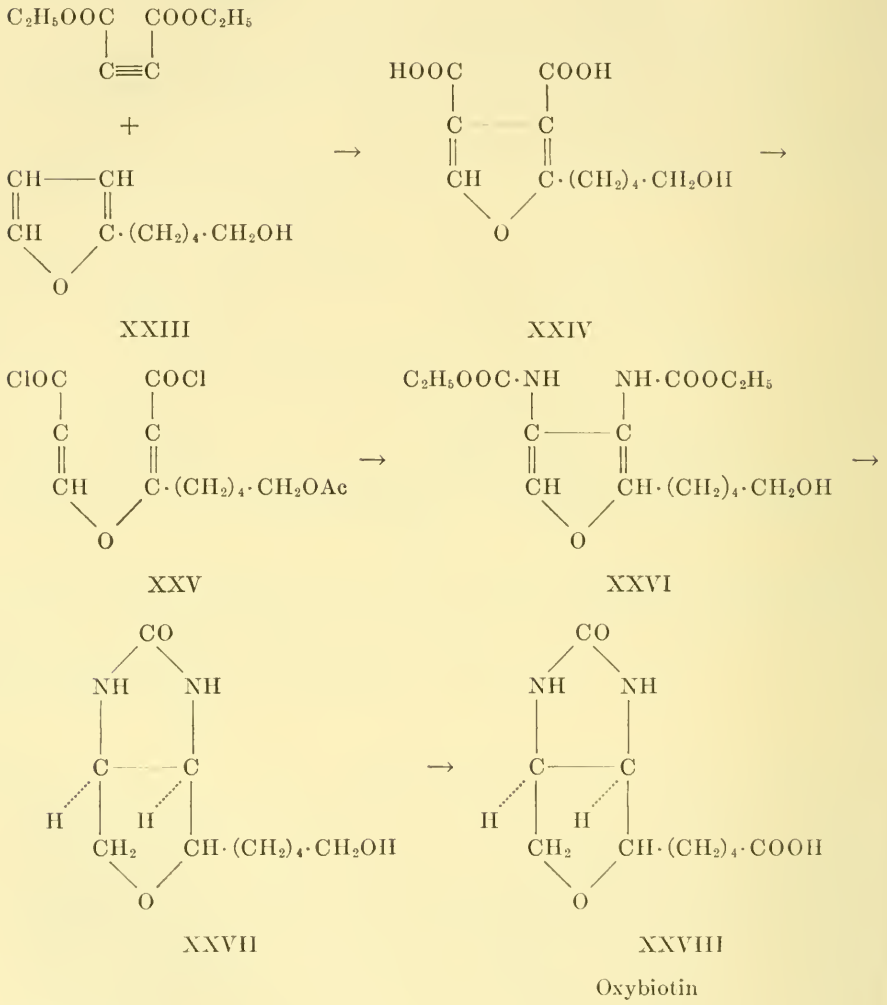


2. Oxybiotin

The synthesis of *dl*-hexahydro-2-oxo-1H-furo[3,4]imidazole-4-valeric acid, the oxygen analog of biotin was reported almost simultaneously by Hofmann[70, 71] and by Duschinsky and his associates.[72] The compound is called oxybiotin[73] or O-heterobiotin.[72] Among the possible stereoismeric forms only the two *cis*-3,4-diamino-2-tetrahydrofuranvaleric acids formed bicyclic urea derivatives. Thus, it appears that the tetrahydrofuran is more planar in its configuration than the tetrahydrothiophene ring, which apparently permits the formation of *trans*-urea derivatives.

[68] G. Swain, *J. Chem. Soc.* **1948**, 1552.
[69] H. McKennis and V. du Vigneaud, *J. Biol. Chem.* **68**, 1507 (1946).
[70] K. Hofmann, *J. Am. Chem. Soc.* **67**, 694 (1945).
[71] K. Hofmann, *J. Am. Chem. Soc.* **67**, 1459 (1945).
[72] R. Duschinsky, L. A. Dolan, D. Flower, and S. H. Rubin, *Arch. Biochem.* **6**, 480 (1945).
[73] F. J. Pilgrim, A. E. Axelrod, T. Winnick, and K. Hofmann, *Science* **102**, 35 (1945).

Hofmann used the following scheme (XXIII–XXVIII) for the synthesis of *dl*-oxybiotin.[71]



A series of homologs of oxybiotin, in which the side chain contained two to six methylene groups,[74] and their sulfonic acid analogs[75-77] were also synthesized.

[74] K. Hofmann, C. Chen, A. Bridgewater, and A. E. Axelrod, *J. Am. Chem. Soc.* **69,** 191 (1947).

[75] A. E. Axelrod and K. Hofmann, *J. Biol. Chem.* **180,** 525 (1949).

[76] K. Hofmann, A. Bridgewater, and A. E. Axelrod, *J. Am. Chem. Soc.* **69,** 1550 (1947).

[77] K. Hofmann, A. Bridgewater, and A. E. Axelrod, *J. Am. Chem. Soc.* **71,** 1253 (1949).

F. COMPLEX BIOTIN COMPOUNDS

In natural products biotin occurs mainly in bound form (p. 529). The biological activity of various tissue extracts in animals and in microorganisms, including yeast cells, shows variations, depending on the form in which biotin was isolated from these sources. Complete hydrolysis will lead to free biotin. One of the simple biotin compounds, the chemical structure of which is now well established, is biocytin.[78-81]

1. Biocytin

The term biocytin (from the Greek *kutos*, meaning cell) has been used to designate the biotin compound occurring in many soluble natural products, especially during the controlled autolysis of actively metabolizing yeast.[78, 80] Biocytin is characterized by its differential availability as source of biotin in microbiological assays using various test organisms. Biocytin may be utilized as biotin by *Lactobacillus casei*, *Lactobacillus delbrückii LD 5*, *Lactobacillus acidophilus*, *Streptococcus fecalis R.*, *Neurospora crassa*, and *Saccharomyces carlsbergensis*. In contrast *Lactobacillus arabinosus*, *Lactobacillus pentosus*, and *Leuconostoc mesenteroides P-60* will respond only to free biotin. Strong acid hydrolysis (at least 3 *N* at 120° for 1 hour) will liberate biotin or its microbiological equivalent from biocytin. No enzymes have been found as yet which will cleave the complex and release biotin.[80, 81] In no biological system thus far studied has biocytin shown more activity than can be accounted for on the basis of its biotin content. During the isolation of biocytin drastic conditions such as extremes of pH, heat, or oxidizing conditions were avoided in order to prevent the formation of artifacts or even of derivatives. Countercurrent distribution in different systems showed only one biotin-containing peak. Finally, crystalline biocytin has a microbiological spectrum or activity identical with that of the biotin complex of yeast extract as shown by bioautographic paper strip chromatography. Thus it appears that biocytin, as isolated from yeast extract, is identical with the compound as it occurs in its natural form.

The isolation of crystalline biocytin from yeast extract involved the following steps:[81] adsorption on Norit; elution with aqueous ammonia; adsorption on Super Filtrol-Celite; elution with aqueous ethanolic ammonia; chromatography on Super Filtrol-Celite; chromatography on alu-

[78] L. D. Wright and H. R. Skeggs, *Proc. Soc. Exptl. Biol. Med.* **56**, 95 (1944).

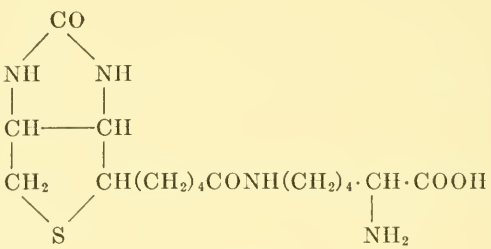
[80] L. D. Wright, E. L. Cresson, H. R. Skeggs, T. R. Wood, R. L. Peck, D. E. Wolf, and K. Folkers, *J. Am. Chem. Soc.* **72**, 1048 (1950).

[80] L. D. Wright, E. L. Cresson, H. R. Skeggs, R. L. Peck, D. E. Wolf, T. R. Wood, J. Valiant, and K. Folkers, *Science* **114**, 635 (1951).

[81] L. D. Wright, E. L. Cresson, H. R. Skeggs, T. R. Wood, R. L. Peck, D. E. Wolf, and K. Folkers, *J. Am. Chem. Soc.* **74**, 1996 (1952).

mina; partition with butanol and cresol; countercurrent distribution; and crystallization from water or from aqueous methanol or aqueous acetone. When the biocytin was allowed to crystallize rapidly from aqueous methanol or acetone the product obtained melted at 228 to 230° (dec. microblock). After slow crystallization from aqueous acetone, biocytin melted at 245 to 252° (dec.).

Acid hydrolysis of recrystallized biocytin yielded about 60% of biotin measured by microbiological assay. The same result was obtained when alkaline hydrolysis was used, followed by reaction with phosgene to convert the biotindiamine to biotin. Microanalysis of the free base gave the composition $C_{16}H_{28}H_4O_4S$. Paper chromatography of the hydrolyzate revealed one ninhydrin-reacting spot, which was identified as lysine by its R_f value. Microbiological assay confirmed the presence of lysine in the hydrolyzate. In biocytin a free amino group was found as evidence by reaction with ninhydrin and 2,4-dinitro-fluorobenzene. Nitrous acid interacts with the lysine, furnishing after hydrolysis a derivative which did not react with ninhydrin under the test conditions. Thus, biocytin appears to be ε-N-biotinyl-L-lysine.[80, 82]



Biocytin

The structure of biocytin was confirmed by its synthesis from synthetic biotin acid chloride[83] and the copper chelate complex of L-lysine. Reaction of biotin acid chloride with α-N-formyl-L-lysine to give ε-N-biotinyl-α-N-formyl-L-lysine, followed by hydrolytic removal of the formyl group, also yielded ε-N-biotinyl-L-lysine.[84]

Biocytin isolated from yeast extract and the synthetic compound were compared by a number of chemical and biological tests and were found in all respects identical.[82, 85] This comparison included infrared absorption spectra, microbiological activities, stimulation of specific enzymatic reac-

[82] R. L. Peck, D. E. Wolf, and K. Folkers, *J. Am. Chem. Soc.* **74**, 1999 (1952).
[83] D. E. Wolf, J. Valiant, and K. Folkers, *J. Am. Chem. Soc.* **73**, 4142 (1951)
[84] D. E. Wolf, J. Valiant, R. L. Peck, and K. Folkers, *J. Am. Chem. Soc.* **74**, 2002 (1952).
[85] L. D. Wright, E. L. Cresson, K. V. Liebert, and H. R. Skeggs, *J. Am. Chem. Soc.* **74**, 2004 (1952).

tions, behavior on paper chromatograms, rates of hydrolysis resistance toward commercial enzymes and inactivation by egg white.

2. Other Forms of Bound Biotin

Free biotin as well as biocytin are water-soluble dialyzable compounds. In contrast, biotin as it occurs in tissues and as it participates in various specific metabolic reactions appears to be bound to protein or to peptides or similar higher molecular compounds, perhaps by peptide linkages.[86] Through enzymatic hydrolysis of liver protein with crystalline pepsin, soluble bound protein compounds were obtained, the chemical nature of which has not yet been elucidated. The purest of these components contained 50 atoms of nitrogen per mole of biotin.[87]

The biotin activity present in acetone-dried beef liver may be almost quantitatively extracted with a buffer solution of pH 3 and dilute NaOH. Two distinct fractions containing biotin were thus obtained. The substance soluble at pH 3 possessed biotin activity for yeast and showed no further increase of activity by acid hydrolysis. This acid-soluble active compound is dialyzable. On the other hand the material extracted with dilute alkali is nondialyzable and exhibits biotin activity only after treatment with acid. It may represent a soluble biotin-protein complex, named biotoprotein.[88]

A "saline-soluble," high-molecular, undialyzable, microbiologically active (for yeast) biotin complex was found in egg yolk.[89]

3. Avidin

The most interesting bound form of biotin is its combination with a special protein-like constituent of egg white named avidin.[90, 91] Avidin represents the "toxic" component of raw egg white responsible for the production of egg white injury (p. 527).

The induced nature of egg white injury was first suggested by experiments in which feces obtained from rats suffering from egg white injury were fed to like animals. The feces were found to be non-potent as source of biotin but were highly active after steaming. It was assumed that some constituent of egg white combines with and holds in non-absorbable form the curative factor which originated either from the diet alone or was excreted into the digestive tract. The non-absorption of the curative factor would then be responsible for the egg white injury.[92]

[86] J. P. Bowden and W. H. Peterson, *J. Biol. Chem.* **178**, 533 (1949).
[87] Chang Wei-Shen and W. H. Peterson, *J. Biol. Chem.* **193**, 587 (1951).
[88] K. Hofmann, D. F. Dickel, and A. E. Axelrod, *J. Biol. Chem.* **183**, 481 (1950).
[89] P. György and C. S. Rose, *Proc. Soc. Exptl. Biol. Med.* **49**, 294 (1942).
[90] R. E. Eakin, E. E. Snell, and R. J. Williams, *J. Biol. Chem.* **136**, 801 (1940).
[91] R. E. Eakin, E. E. Snell ,and R. J. Williams, *J. Biol. Chem.* **140**, 535 (1941).
[92] H. T. Parsons, J. Gardner, and C. T. Walliker, *J. Nutrition* **19**, *Suppl.* 19 (1940).

By the microbiological method it has been demonstrated[93] that the tissues of chicks on a diet causing egg white injury were deficient in biotin, despite the abundance of this vitamin in the diet. Further it has been shown[90] that commercial or fresh egg white is capable of inactivating biotin *in vitro*, owing to the formation of a fairly stable compound of biotin with a special constituent of egg white. This compound, avidin, has been purified and obtained in crystalline form[91, 94] By definition one unit of avidin combines with 1 γ of biotin. With the assumption that one molecule of biotin combines with one molecule of protein, the molecular weight of avidin has been calculated to be 43,500. Crystalline avidin is slightly less active than highly purified amorphous preparations: 4000 units per gram as compared with 7000 units per gram. The isoelectric point of avidin is claimed to be at pH 10.[95]

Avidin, or egg white containing avidin, is denatured and inactivated by heat. This explains why egg white injury may be produced only with raw or slightly cooked but not with heat-denatured egg white.

Purified avidin produced effects in rats similar to those caused by dried egg white.[96] The avidin-biotin complex (AB) is easily split by steaming for a short time (30 to 60 minutes) at 100°. Rats fed a diet contained cooked egg white plus avidin excreted only negligible amounts of free biotin, whereas after the feces had been steamed large additional amounts of biotin became free.[97]

Solutions of purified avidin lose activity on standing, more readily in dilute than in concentrated solution. In any concentration the rate of destruction is much greater at 38° than at ice-box temperature, and, except in the most concentrated solutions, even room temperature will cause considerable acceleration of the rate. Dilute solutions of egg white are much less affected over this temperature range.[98] When solutions of egg white[11] or of avidin concentrates were brought to pH 1.8 with HCl, their avidin activity was almost but never quite completely destroyed. The avidin-biotin complex is much more stable than avidin itself. Biotin already bound to the avidin was not released by the treatment with acid[98] or trypsin, pancreatin, and papain, or with liver, kidney, muscle, and blood.[99] The high resistance of the avidin-biotin complex to enzymes might contribute to the fact that it passes through the alimentary tract practically unchanged.

[93] R. E. Eakin, W. A. McKinley, and R. J. Williams, *Science* **92**, 224 (1940).
[94] D. Pennington, E. E. Snell, and R. E. Eakin, *J. Am. Chem. Soc.* **64**, 469 (1942).
[95] D. W. Woolley and L. G. Longsworth, *J. Biol. Chem.* **142**, 285 (1942).
[96] P. György, C. S. Rose, R. E. Eakin, E. E. Snell, and R. J. Williams, *Science*, **93**, 477 (1941).
[97] P. György and C. S. Rose, *Science* **94**, 261 (1941).
[98] P. György, C. S. Rose, and R. M. Tomarelli, *J. Biol. Chem.* **144**, 169 (1942).
[99] P. György and C. S. Rose, *Proc. Soc. Exptl. Biol. Med.* **53**, 55 (1943).

Irradiation with visible light inactivates avidin in solutions of egg white or of purified concentrates, the latter more easily. Biotin bound to the avidin may be released. The presence of riboflavin accelerates the action of visible light.[98] Biotin could also be liberated from the avidin-biotin complex by oxidation with 0.45% H_2O_2.

By means of radioactive biotin the reaction between avidin and biotin was found to be practically complete but to some extent reversible with a measurable dissociation of the complex.[100] The radiobiotin was prepared with radioactive C_{14} in the ureido position.[101] On the basis of equilibrium measurements, it was found that an expression of the type $K = \frac{(A)(B)^2}{AB_2}$ with a modification for the incomplete dissociation of the intermediate AB describes the system over a considerable range of concentrations. An average value was found for $K = 0.6 \times 10^{-21}$, at 25° in 0.2 M ammonium carbonate and other media, corresponding to a degree of dissociation of 0.08% at 10^{-6} M complex. The dissociation was found to increase with temperature and seems to increase in certain specific media.

The technique of Sanger[102, 103] has been applied to avidin for the characterization and determination of amino groups. It has been shown that in avidin preparations, free from nucleoprotein and nucleic acid, all the lysine ϵ-amino groups[25] react and that there are three N-terminal alanine residues per mole.[104]

Avidin which is toxic when given enterally is of high therapeutic value in egg white injury when administered parenterally.[97] This is explained by liberation of the biotin consistently present in avidin concentrates. The mechanism of the splitting of the avidin-biotin complex in a parenteral medium has not yet been explained. It is certainly at variance with its stability in the intestinal tract, and it may be related to oxidative, perhaps fermentative, reactions in the parenteral medium.[99]

The only source of avidin so far known is egg white (hen, turkey, duck, and goose) and also the egg jelly of frogs. It is from the oviduct tissue, probably from its mucosal lining, that biotin-binding avidin is secreted in hen and frog.[105] No explanation has been found for the presence of this antivitamin in the oviduct and in the egg.

The avidin combinability of biotin, its analogs, and related compounds has been used for their chemical characterization. The "relative affin-

[100] H. F. Launer and H. Fraenkel-Conrat, *J. Biol. Chem.* **193**, 125 (1951).
[101] D. B. Melville, J. G. Pierce, and C. W. H. Partridge, *J. Biol. Chem.* **180**, 299 (1949).
[102] F. Sanger, *Biochem. J.* **39**, 507 (1945).
[103] R. R. Porter and F. Sanger, *Biochem. J.* **42**, 287 (1948).
[104] H. Fraenkel-Conrat and R. R. Porter, *Biochem. J.* **49**, lxxviii (1951).
[105] R. Hertz and W. H. Sebrell, *Science* **96**, 257 (1942).

ity"[106, 107] is expressed as the ratio of the concentration of analog to biotin at which one-half of the biotin remains free and available for growth of the test organism. Thus, the ratio will be low for analogs for which avidin has considerable affinity and high for those analogs that do not combine readily with avidin. With this technique, synthetic (−)-biotin and *dl*-allobiotin have shown no significant affinity for avidin. The affinity ratios for biocytin,[84] *dl*-epiallobiotin, *dl*-desthiobiotin, and δ-(2,3-ureylenecyclohexyl)valeric acid were 3, 6, 10, and 14, respectively. γ-(3,4-Ureylenecyclohexyl)butyric acid and δ-(3,4-ureylenecyclohexyl)valeric acid have shown definite but very low affinity for avidin.

Among other analogs the following have been found to combine with avidin: *dl*-oxybiotin, *dl*-oxybiotin methyl ester, *dl*-hexahydro-2-oxo-1H-furo[3,4]imidazole-4-pentanol,[108] and 2-oxo-4-imidazolidine valeric, caproic, enanthic, and caprylic acids.[109]

The inability of *cis*-3,4-diamino-2-tetrahydrothiophenevaleric acid (the diaminocarboxylic acid corresponding to biotin), ζ,η-diaminopelargonic acid (an analog of desthiobiotin), and *dl-cis*-3,4-diamino-2-tetrahydrofuraurvaleric acid (corresponding to oxybiotin) to combine with avidin[108-110] appears to confirm the assumption that avidin combinability is a function of the imidazolidine ring. Since desthiobiotin and other similar sulfur-free derivatives as well as the methyl ester of oxybiotin or the alcohol analog of oxybiotin combine with avidin, neither the sulfur atom nor the carboxyl group of the biotin molecule is essential for the bonding with avidin.[108, 109]

Some of the biotin is excreted in the urine of normal rats and in human urine as avidin-uncombinable fractions.[111, 112] The chemical nature of the biotin derivatives in the urine, termed miotin, tiotin, and rhiotin,[113] which—it is claimed—differ from each other by their resistance to heat, avidin combinability, and varying activity for yeast and *Rhizobium*, has not yet been determined.

G. SPECIFICITY

1. Biotin

The configuration of the biotin molecule and of its derivatives determines the specificity of their action for animals and microorganisms.

[106] L. D. Wright and H. R. Skeggs, *Arch. Biochem.* **12**, 27 (1947).

[107] L. D. Wright, H. R. Skeggs, and E. L. Cresson, *Proc. Soc. Exptl. Biol. Med.* **64**, 150 (1947).

[108] T. Winnick, K. Hofmann, F. J. Pilgrim, and A. E. Axelrod, *J. Biol. Chem.* **161**, 405 (1945).

[109] K. Dittmer and V. du Vigneaud, *Science* **100**, 129 (1944).

[110] V. du Vigneaud, K. Dittmer, K. Hofmann, and D. B. Melville, *Proc. Soc. Exptl. Biol. Med.* **50**, 374 (1942).

[111] T. W. Oppel, *Am. J. Med. Sci.* **204**, 886 (1942).

[112] E.-J.-H. Chu and R. J. Williams, *J. Am. Chem. Soc.* **66**, 1678 (1944).

[113] D. Burk and R. J. Winzler, *Science* **97**, 57 (1943).

Table I[114] contains the relevant data for the various biotin isomers. *d*-Biotinol,[118] the alcohol analog of biotin, is inactive for *Lactobacillus arabinosus*, *L. casei*, and *Saccharomyces cerevisiae* but is fully as effective as *d*-biotin in curing egg-white-induced biotin deficiency in the rat. Excretion studies in rats and man showed that biotinol after oral or intramuscular administration is effectively converted to biotin.[119]

Filtration of bacteriological culture media through Seitz filters should take in consideration the possibility that such Seitz filters may release biotin originating from previous contamination.[120]

It is of interest that, of the two racemic diastereoisomers of the oxygen

TABLE I

Specific Activity of Biotin Isomers[114]

Compound	Organism	Activity	Reference
(+)-Biotin		100	
(−)-Biotin	Rat	0 (<13)	115
	Chick	0	116
	Lactobacillus casei	0.023	117
	Lactobacillus arabinosus	0.006–0.019	117
dl-Biotin	Rat	50	115
	Chick	ca. 50	116
	Lactobacillus casei	ca. 50	117
	Lactobacillus arabinosus	ca. 50	62, 117
	Saccharomyces cerevisiae	ca. 50	62
dl-Epibiotin	*Saccharomyces cerevisiae*	0	53
dl-Allobiotin	Rat	0	115
	Chick	0	116
	Lactobacillus casei	0.0029	117
	Lactobacillus arabinosus	0.002	117
dl-Epiallobiotin	*Lactobacillus arabinosus*	0	51, 52

analog of biotin, only one, *dl*-oxybiotin,[121] possesses appreciable activity (Table II). The other, *dl*-epioxybiotin, is practically inactive. Its very slight activity for *Lactobacillus arabinosus* (0.1 % that of oxybiotin) is probably caused by oxybiotin as a contaminant.[131] It is to be expected that, as with biotin, only one of the optically active forms of oxybiotin is

[114] R. J. Williams, R. E. Eakin, E. Beerstecher, Jr., and W. Shive, The Biochemistry of B Vitamins. Reinhold Publishing Corp., New York, 1950.

[115] G. A. Emerson, *J. Biol. Chem.* **157**, 127 (1945).

[116] W. H. Ott, *J. Biol. Chem.* **157**, 131 (1945).

[117] J. L. Stokes and M. Gunness, *J. Biol. Chem.* **157**, 121 (1945).

[118] M. W. Goldberg and L. H. Sternbach, U. S. Pat. 2,489,237.

[119] L. Drekter, J. Scheiner, E. DeRitter, and S. H. Rubin, *Proc. Soc. Exptl. Biol. Med.* **78**, 381 (1951).

[120] S. N. Basu and R. G. Bose, *Nature* **165**, 851 (1950).

[121] K. Hofmann and A. E. Axelrod, *Arch. Biochem.* **11**, 375 (1946).

biologically active. For *L. arabinosus* and *L. pentosus* 124-2 oxybiotin and biotin are equally active. For other organisms oxybiotin is often less effective in obtaining the maximum response.

The activity of oxybiotin appears to be dependent upon the pH of the medium. For *Streptococcus faecalis* it is essentially inactive at pH 6.6 and shows some, although weak, activity at pH 7.3.[129] The growth of *Lactobacillus arabinosus* at pH 6.5 is not more than one-fifth of the maximum, regardless of the level of oxybiotin. When the initial pH is 7.0 or more, excellent growth results and oxybiotin is about 30 % as active as biotin.[130]

TABLE II

THE BIOLOGICAL ACTIVITY OF *dl*-OXYBIOTIN[114]

Organism	Activity of (+)-biotin, %	Reference
Rat	6.0–2.0[a, b]	122–124
Chicks	17[b]	122, 125
	20–2[b, c]	126
Lactobacillus arabinosus	50	73, 127
Lactobacillus pentosus 124-2	50	128
Lactobacillus casei	40	73, 108
	22–25	72, 124
Streptococcus faecalis R.	0 (pH 6.6)	129
	7.5 (pH 7.3)	129
Rhizobium trifolii	12–1.3[b]	108
Saccharomyces cerevisiae	25–10[b]	72, 73, 124, 108
Saccharomyces carlsbergensis	20[a]	124

[a] Biotin deficiency induced by feeding raw egg white with biotin-deficient diet; *dl*-oxybiotin injected intramuscularly or subcutaneously.

[b] The dose-response curve differs in shape for (+)-biotin and oxybiotin. The relative activity of oxybiotin tends to decrease with increases in concentrations.

[c] Animals on biotin deficient diet only.

In rats and chicks the specific signs of biotin deficiency may be completely cured by oxybiotin.[123, 125] However, in the rat the general shape of the dose-

[122] K. Hofmann, R. H. McCoy, J. R. Felton, A. E. Axelrod, and F. J. Pilgrim, *Arch. Biochem.* **7**, 393 (1945).

[123] A. E. Axelrod, F. J. Pilgrim, and K. Hofmann, *J. Biol. Chem.* **163**, 191 (1946).

[124] S. H. Rubin, D. Flower, F. Rosen, and L. Drekter, *Arch. Biochem.* **8**, 79 (1945).

[125] R. H. McCoy, J. R. Felton, and K. Hofmann, *Arch. Biochem.* **9**, 141 (1946).

[126] P. R. Moore, T. D. Luckey, C. A. Elvehjem, and E. B. Hart, *Proc. Soc. Exptl. Biol. Med.* **61**, 185 (1946).

[127] D. B. Melville, K. Dittmer, G. B. Brown, and V. du Vigneaud, *Science* **98**, 497 (1943).

[128] K. K. Krueger and W. H. Peterson, *J. Bacteriol.* **55**, 693 (1948).

[129] T. D. Luckey, P. R. Moore, and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.* **61**, 97 (1946).

[130] R. L. Potter and C. A. Elvehjem, *J. Biol. Chem.* **183**, 587 (1950).

response curve for oxybiotin seems to be different from that for biotin.[123] The relative activity for oxybiotin is higher for a suboptimal (6.0%) than for near maximal (2.9%) response. For chicks it was claimed that oxybiotin injected intramuscularly was approximately 17% as active as (+)-biotin with the shape of the dose-response curve essentially similar for both compounds.[125] In contrast, the statement was also made that oxybiotin, when administered as a supplement in the diet instead of being given parenterally, fails to replace biotin completely for growth of the chick on a biotin-deficient diet. Oxybiotin is reported to have about one-third the activity of biotin in curing the specific cutaneous signs of biotin deficiency. However, even with up to 100 γ per 1000 g. of diet, oxybiotin failed to support optimal growth in chicks. Thus it was assumed—without confirmation—that oxybiotin, at least when given in the food, fulfilled only a part of the function of biotin in the chick.[108]

There are several methods available for the differential assay of biotin and oxybiotin, respectively. Whereas Raney nickel[132] or oxidation with permanganate[133] will destroy biotin, the same procedures leave oxybiotin unaffected. Raney nickel converts biotin to desthiobiotin and permanganate oxidizes biotin to corresponding sulfone. Neither of these compounds has any appreciable activity for *Lactobacillus arabinosus*, as well as for several other organisms. Thus, in such mixtures oxybiotin may be quantitatively determined as a growth factor for *L. arabinosus* and similar organisms.

Inasmuch as *Streptococcus faecalis R.* is unable to utilize oxybiotin effectively, especially at low pH when it still responds to biotin, *S. faecalis* may be used for the quantitative estimation of biotin with only negligible interference from the oxygen analog.[129]

A further indirect assay of biotin in the presence of oxybiotin depends on the inhibitory effect of biotin sulfone or γ-(3,4-ureylene-cyclohexyl)butyric acid in the response of *Lactobacillus arabinosus* for moderate amounts of oxybiotin.[134] Under these conditions, neither of the inhibitors appreciably affects the utilization of biotin by the organism.

With these assays, oxybiotin has been found not to occur naturally in any of the organisms or animal tissues tested. On the other hand, it has also been demonstrated that *Saccharomyces cerevisiae*, *Rhizobium trifolii*, and *Lactobacillus pentosus* grown in the presence of oxybiotin utilized the compound as such, and not after conversion into biotin.[128, 133, 135] Various tissues taken from biotin-deficient chicks treated with oxybiotin have yielded low values for biotin and appreciable amounts of the oxygen analog.

[131] K. Hofmann, *J. Am. Chem. Soc.* **71**, 164 (1949).
[132] K. Hofmann, T. Winnick, and A. E. Axelrod, *J. Biol. Chem.* **169**, 191 (1947).
[133] K. Hofmann and T. Winnick, *J. Biol. Chem.* **160**, 449 (1945).
[134] A. E. Axelrod, J. DeWoody, and K. Hofmann, *J. Biol. Chem.* **163**, 771 (1946).
[135] A. E. Axelrod, B. C. Flin, and K. Hofmann, *J. Biol. Chem.* **169**, 195 (1947).

Thus, oxybiotin, although not a natural product, may replace biotin in metabolic reactions, and the sulfur atom is not essential for biological activity.

2. Desthiobiotin

This sulfur-free analog of biotin is just as active in the biotin assay with *Saccharomyces cerevisiae* as (+)-biotin, and the dose-response curves were essentially identical.[127] In contrast, desthiobiotin not only was inactive for *Lactobacillus casei* but inhibited competitively the response of the organism to biotin.[136, 137]

Table III contains the available data for the growth-promoting activities of desthiobiotin and other analogs of biotin and oxybiotin.[114]

It appears that organisms which utilize desthiobiotin may convert it into substances which possess biotin-like activity for organisms which require biotin but are unable to utilize desthiobiotin.[117, 136, 145] The biotin activity of cells of *Saccharomyces cerevisiae* grown on either biotin or desthiobiotin may be destroyed with permanganate[133] or with Raney nickel for *Lactobacillus casei*, *L. arabinosus*, and *Rhizobium trifolii*. Thus, it may not be related to oxybiotin. On the other hand, the biotin activity of the compound extracted from *S. cerevisiae* grown in the presence of desthiobiotin for *L. casei* indicates that it is due to biotin itself.

Desthiobiotin had the same activity as biotin for *Neurospora crassa*, *Escherichia coli* 58, and *Penicillium notatum* 21464, but was inactive for an x-ray induced biotin-less mutant of *P. chrysogenum* 62078.[143] This last organism apparently synthesizes and accumulates desthiobiotin, and the addition of pimelic acid enhanced its intracellular production.

From these observations it may be concluded that desthiobiotin, in contrast to oxybiotin, is a normal precursor or is converted to a normal precursor of biotin by a number of organisms.

Among the other biotin analogs the methyl ester of biotin is inactive for *Lactobacillus casei* but fully active for yeast.

Biotin sulfone stimulates the growth of yeast, but even with large

[136] K. Dittmer, D. B. Melville, and V. du Vigneaud, *Science* **99**, 203 (1944).
[137] V. G. Lilly and L. H. Leonian, *Science* **99**, 205 (1944).
[138] S. H. Rubin, L. Drekter, and E. H. Moyer, *Proc. Soc. Exptl. Biol. Med.* **58**, 352 (1945).
[139] G. M. Shull, B. L. Hutchings, and W. H. Peterson, *J. Biol. Chem.* **142**, 913 (1942).
[140] V. du Vigneaud, *Chem. Eng. News* **23**, 620 (1945).
[141] K. Dittmer, V. du Vigneaud, P. György, and C. S. Rose, *Arch. Biochem.* **4**, 229 (1944).
[142] G. B. Brown and V. du Vigneaud, *J. Biol. Chem.* **163**, 761 (1946).
[143] E. L. Tatum, *J. Biol. Chem.* **160**, 455 (1945).
[144] J. A. Hijner, *Experientia* **2**, 312 (1946).
[145] L. H. Leonian and V. G. Lilly, *J. Bacteriol.* **49**, 291 (1945).

amounts of sulfone the yeast growth did not increase above a relatively low maximum which was only about one-third of the maximum growth obtained with biotin. Biotin sulfone was found to be approximately 0.1 %

TABLE III[114]

GROWTH-PROMOTING ACTIVITIES OF ANALOGS OF BIOTIN AND OXYBIOTIN

Compound	Organism	Activity of (+)-biotin, %	References
(+)-Desthiobiotin[a]	*Saccharomyces cerevisiae*	100	127
	Lactobacillus casei	0[b]	127
dl-Desthiobiotin	*Saccharomyces cerevisiae*	60–50	66, 138
	Rats	0.1–0.01	115, 138
dl-Desthioallobiotin	*Saccharomyces cerevisiae*	0	51, 52
Biotin methyl ester	*Saccharomyces cerevisiae*	100	5
	Lactobacillus casei	0	139
dl-Oxybiotin methyl ester	*Saccharomyces cerevisiae*	16–10	108
Biotin sulfoxide methyl ester	*Saccharomyces cerevisiae*	100 ca.	140
	Lactobacillus casei	0	140
Biotin sulfone	*Saccharomyces cerevisiae*	0.1[c]	28, 109, 141
	Rats	0.1	141
	Lactobacillus casei	0	109
cis-3,4-Diamino-2-tetrahydrothiophenevaleric acid	*Saccharomyces cerevisiae*	10	27, 110
	Saccharomyces cerevisiae F.B.	0	112
	Lactobacillus casei	0 (<0.01)	109
	Rats	0 (<1)	115, 141
dl-cis-3,4-Diamino-2-tetrahydrothiophenevaleric acid	*Lactobacillus arabinosus*	1.8	117
	Lactobacillus casei	3.6	117
	Rats	0 (0.4)	115
dl-cis-3,4-Diamino-2-tetrahydrofuranvaleric acid	*Lactobacillus arabinosus*	0.01	108
	Lactobacillus casei	0.35–0.5	108
dl-Hexahydro-2-oxo-1H-furo-[3,4]imidazole-4-pentanol	*Lactobacillus arabinosus*	0.13	108
	Lactobacillus casei	0.07–0.03	108
dl-Hexahydro-2-oxo-4-methyl-1H-furo[3,4]imidazole	*Saccharomyces cerevisiae*	0.0001	108
	Lactobacillus arabinosus	0.0001	108
	Lactobacillus casei	0.0001	108
(+)- ζ,η-Diaminopelargonic acid	*Saccharomyces cerevisiae*	10	39, 109
	Lactobacillus casei	0	39, 109
dl-5-Methyl-2-thiono-4-imidazolidinecaproic acid	*Saccharomyces cerevisiae*	0.06	142
	Lactobacillus casei	0[b]	142

[a] Active for *Saccharomyces cerevisiae* (25 strains), *S. chodati*, *S. macedoniensis*, *Endomycopsis fibuliger* *Debaromyces matruchoti* v. *subglobosus*, *Mycoderma valida*, *Mycotorula lactis*, *Schizosaccharyomyces pombe*, *Torula lactosa*, *Zygosaccharomyces lactis*, *Z. marxianus*, *Neurospora crassa*, *N. sitophila*, *Ceratostomella*, ips 438, *Ceratostomella montium*, *Leuconostoc mesenteroides*, *Penicillium notatum* 21464, *Escherichia coli* 58, *Ceratostomella reukanfi*, *Schwanniomyces occidentalis* 116.[117, 127, 143, 144] Inactive for *Ceratostemella pini* 416, *Sordaria fimicola*, *Lactobacillus arabinosus*, *Rhizobium trifolii* 205,[127] and *Penicillium chrysogenum* 62078.[117, 127]

[b] Compound competitively inhibits utilization of biotin for this organism.

[c] Only 35 to 50% of optimal growth obtained with biotin is attained.

as effective as biotin when the activities were compared at the level of one-fourth maximum growth increase due to biotin. The same ratio (0.1 %) appears to apply also to the curative effect of biotin sulfone on rats with egg white injury.

The stimulation of yeast growth by biotin sulfone is dependent on the presence of aspartic acid in the medium. Asparagine could not be substituted for aspartic acid.[141]

The guanido analogs of *d*-biotin and *dl*-oxybiotin have been synthesized by treatment of the respective *cis*-diaminocarboxylic acid with cyanogen bromide. These compounds were practically devoid of biological activity for *Lactobacillus casei* and *L. arabinosus*, as well as for *Saccharomyces cerevisiae*, thus emphasizing the essential character of the cyclic urea portion for the biological activity of biotin and oxybiotin.[146]

Autoclaving of extremely dilute solutions of biotin with hydrochloric acid but not with sulfuric acid resulted in almost complete loss of its growth-promoting potency for *Lactobacillus casei* and *Streptococcus faecalis* and approximately 50% of *L. arabinosus* and *S. cerevisiae*. It has been assumed[147] that this partial loss of potency of biotin was due to its conversion under the influence of heat and HCl into a mixture of biotin sulfoxide and biotin sulfone. Biotin sulfoxide is as active as biotin (Table III) for *L. arabinosus* and *S. cerevisiae* and inactive for *L. casei* or *S. fecalis*, as well as well as for rats.[140, 147, 148] In contrast, biotin sulfone is practically inactive for all four organisms and also for rats.[109, 141, 147] Many natural products are capable of preventing the conversion of biotin to its sulfoxide and sulfone through the action of hydrochloric acid. Such a "protection" is not found in grass juice.[147]

The conversion of biotin into sulfone via sulfoxide may also be accomplished by H_2O_2[28, 31] and even by rancid oils and fats with a high peroxide content.[149]

With hydrogen peroxide biotin has been found to give a mixture of two diastereoisomeric biotin sulfoxides, the *dextro* form predominating.[148] This form has also been isolated from milk. The *levo* isomer of biotin sulfoxide has been isolated from *Aspergillus niger* culture filtrates.[148a] Since none of the *dextro* isomer was detectable, it is presumed that biotin *l*-sulfoxide is the product of enzymatic reactions.

3. Unspecific Stimulatory Precursors and Substitutes

a. Pimelic Acid

The growth of *Corynebacterium diphtheriae* is stimulated by pimelic acid.[150, 151] In some strains of this organism pimelic acid appears to be used for the synthesis of biotin. For such strains biotin not only replaces pimelic acid but is found to be more effective than pimelic acid, especially at low concentrations.[152]

b. Oleic Acid and Related Compounds

Oleic acid and related compounds, such as lecithin, may, in the presence of aspartic acid, effectively replace biotin for *Lactobacillus casei* and certain other lactobacilli, even for yeast, for which biotin constitutes an es-

[146] K. Hofmann and A. E. Axelrod, *J. Biol. Chem.* **187**, 29 (1950).
[147] A. E. Axelrod and K. Hofmann, *J. Biol. Chem.* **187**, 23 (1950).
[148] D. B. Melville, D. S. Genghof, and J. M. Lee, *Federation Proc.* **9**, 204 (1950); *J. Biol. Chem.* In press; D. B. Melville, *J. Biol. Chem.* In press.
[148a] L. D. Wright and E. L. Cresson, *J. Am. Chem. Soc.* In press; L. D. Wright, E. L. Cresson, J. Valiant, D. E. Wolf, and K. Folkers, *ibid.* In press; L. D. Wright, E. L. Cresson, J. Valiant, D. E. Wolf, and K. Folkers, *ibid.* In press.
[149] P. L. Pavcek and G. M. Shull, *J. Biol. Chem.* **146**, 351 (1942).
[150] J. H. Mueller, *Science* **85**, 502 (1937).
[151] J. H. Mueller, *J. Biol. Chem.* **119**, 124 (1937).
[152] V. du Vigneaud, K. Dittmer, E. Hague, and B. Long, *Science* **96**, 186 (1942).

sential nutrient in the medium.[153-157] This so-called oleic acid factor may even satisfy the biotin requirement of mosquito larvae (*Aëdes aegypti*) for their growth to the fourth instar.[158]

The *cis*-octadecenoic acids to which the oleic acid (*cis*-9-octadecenoic acid) belongs showed in test no great differences in biotin-like activity. In contrast, wide variations were found among the *trans* acids. The activity of *trans*-9-octadecenoic (elaidic) acid was of the same order as that of oleic acid. Activity of *trans* acids tested decreased for positional change of the double bond on either side of *trans*-9- to almost no activity for *trans*-6- and for Δ-17-octadecenoic acid. Considerable synergistic activity with biotin was noticeable for the *trans* compounds except for *trans*-9-octadecenoic acid, but only additive effect for the *cis* acids. The biotin-like effect of isoöleic acid mixtures formed during hydrogenation of vegetable oils was comparable to that of oleic acid itself. Saturated fatty acids, without showing any biotin-like activity *per se*, augmented the activity of the unsaturated fatty acids in the mixture.[159]

The dextran-forming *Leuconostoc* requires biotin in glucose and fructose media but does not require this vitamin in sucrose media to the degree that the disaccharide was utilized via the mechanism resulting in dextran synthesis.[160] Biotin may be replaced for *Leuconostoc* by oleic acid, which was used in the form of the surface-active polyoxyethylene sorbitan monoöleate (Tween 80) and even by the saturated fatty acid, lauric acid, when added to the medium in form of Tween 20, the surface active polyoxyethylene sorbitan monolaurate.[161]

Various explanations were given for the biotin-sparing effect of oleic acid and related compounds. It has been postulated that biotin is essential for the synthesis of oleic acid and perhaps of other fatty acids[155] or that oleic acid may act as a precursor or should function only by alteration of cell permeability.[153, 154] The effect of Tween 20 on *Leuconostoc* underscores the physical factor. On the other hand it has been recently shown that washed cells of *Lactobacillus casei* harvested from the culture medium containing oleic acid and no biotin contained ten times as much biotin as originally

[153] V. R. Williams and E. A. Fieger, *J. Biol. Chem.* **166**, 335 (1946).
[154] V. R. Williams and E. A. Fieger, *J. Biol. Chem.* **170**, 399 (1947).
[155] W. L. Williams, H. P. Broquist, and E. E. Snell, *J. Biol. Chem.* **170**, 619 (1947).
[156] A. E. Axelrod, K. Hofmann, and B. F. Daubert, *J. Biol. Chem.* **169**, 761 (1947).
[157] A. E. Axelrod, M. Mitz, and K. Hofmann, *J. Biol. Chem.* **175**, 265 (1948).
[158] W. Trager, *J. Biol. Chem.* **176**, 1211 (1949).
[159] A. L. S. Cheng, S. M. Greenberg, H. J. Deuel, Jr., and D. Melnick, *J. Biol. Chem.* **192**, 611 (1951).
[160] W. W. Carlson and V. Whiteside-Carlson, *Proc. Soc. Exptl. Biol. Med.* **71**, 416 (1949).
[161] W. W. Carlson, V. Whiteside-Carlson, and K. Kospetos, *Federation Proc.* **9**, 159 (1950).

present in the total volume of the medium. This finding speaks in favor of biotin synthesis in the presence of oleic acid.[162]

Many lactic acid bacteria require both biotin and oleic acid for optimal growth.[155, 163-165]

It was claimed[166] that the intramuscular injection of a fat-soluble fraction (FSF)—a neutral oil—obtained from hydrolyzed plasma into chicks fed a diet high in egg white reduced the severity of their dermatitis. The similar injection of oleic acid did not have such an effect, nor could the effect be produced by the oral administration of the material from plasma. Through countercurrent distribution and by chromatographic adsorption, fractions were prepared which showed as high a specific activity for *Lactobacillus casei* as oleic acid but which differed from oleic acid in physical properties.[166] The chemical nature of the active substance has not been determined. Since on prolonged heating with alkali the activity of this fat-soluble fraction from plasma goes into the saponifiable fraction and behaves toward *Lactobacillus casei* as oleic acid,[167] it is highly probable that oleic acid represents its specific active principle, perhaps in a less toxic form than free oleic acid.

c. Inhibitory Analogs

Several analogs of biotin may have an inhibitory effect, usually on a competitive basis, on the utilization of biotin or other biotin compounds by various organisms. The inhibitory activities of a compound are given in terms of the molar inhibition ratio,[109] which has been defined as the number of molecules of the inhibitor necessary to prevent the biological effect of one molecule of biotin. The molar inhibition ratio[109] of a given inhibitor to biotin is obtained experimentally by determining the amount of the inhibitor required to reduce the growth obtained with 0.0002 γ of biotin to the level of growth obtained with 0.0001 γ of biotin. The inhibition index just necessary for complete inhibition of growth is usually greater than that obtained for half-maximum inhibition of growth.

d. Desthiobiotin and Related Compounds

Desthiobiotin stimulates the growth of *Saccharomyces cerevisiae* in a biotin-free medium. On the other hand it will competitively interfere with the utilization of biotin by *Lactobacillus casei*[136, 137] (p. 562). Several other analogs of desthiobiotin were prepared and tested for their inhibitory

[162] E. A. Andrews and V. R. Williams, *J. Biol. Chem.* **193,** 11 (1951).
[163] B. L. Hutchings and E. Boggiano, *J. Biol. Chem.* **169,** 229 (1947).
[164] A. R. Whitehill, J. J. Oleson, and Y. SubbaRow, *Arch. Biochem.* **15,** 31 (1947).
[165] B. M. Guirard, E. E. Snell, and R. J. Williams, *Arch. Biochem.* **9,** 361 (1946).
[166] W. Trager, *Proc. Soc. Exptl. Biol. Med.* **64,** 129 (1947).
[167] K. Hofmann and A. E. Axelrod, *Arch. Biochem.* **14, 482** (1947).

activity not only in relation to biotin but also to oxybiotin and desthiobiotin. In replacing biotin with oxybiotin or desthiobiotin, amounts of the compounds biologically equivalent to the indicated amounts of biotin are used for determining the molar inhibition ratios. The available data are summarized in Table IV.

It is of interest that the inhibitory effect of desthiobiotin appears to be due only to the dextrorotatory form which is also the only form which exerts a growth-promoting activity for *Saccharomyces cerevisiae*. Oxybiotin may overcome the toxicity of desthiobiotin for *Lactobacillus casei*, but it is considerably less effective than biotin. The results indicate that in the competition for the enzyme involved the affinity of oxybiotin is much lower than that of biotin.

Some of the inhibitory analogs tested represent modifications of desthiobiotin in which the length of the side chain containing the carboxyl group is varied, the 5-methyl group is omitted, or the carboxyl group is replaced by a sulfonic acid group (Table IV). Among these analogs, *dl*-2-oxo-4-imidazolidinecaproic acid proved to be especially potent in preventing the utilization of desthiobiotin for *Escherichia coli*. Since in this case biotin prevents the inhibition non-competitively, the analog appears to prevent the conversion of desthiobiotin to biotin. In other instances, the utilization of both compounds, biotin and desthiobiotin, may be inhibited.

The claim that desthiobiotin may have an inhibitory effect on the growth of tumors[173] has not yet been confirmed.

c. Homologs and Sulfone Analogs of Biotin

The most potent inhibitors are biotin sulfone, *dl*-homobiotin, and their various analogs (Table V). (In homobiotin the side chain contains 5; in norbiotin, 3 (CH_2).)

f. Analogs of Oxybiotin

The homologs of oxybiotin and related compounds are practically inactive as inhibitors for biotin but show marked activity in preventing the utilization of oxybiotin (Table VI).

The fermentation ratio of biotin-deficient yeast cells is stimulated in the presence of ammonium sulfate by biotin or oxybiotin. *dl*-Homoöxybiotin or the sulfonic acid analog of oxybiotin will prevent the fermentation

[168] K. Dittmer and V. du Vigneaud, *J. Biol. Chem.* **169**, 63 (1947).
[169] R. Duschinsky and L. A. Dolan, *J. Am. Chem. Soc.* **68**, 2350 (1946).
[170] L. L. Rogers and W. Shive, *J. Biol. Chem.* **169**, 57 (1947).
[171] H. McKennis, Jr., and V. du Vigneaud, *J. Am. Chem. Soc.* **68**, 832 (1946).
[172] R. Duschinsky and S. H. Rubin, *J. Am. Chem. Soc.* **70**, 2546 (1948).
[173] J. C. Keresztesy, D. Laszlo, and C. Leuchtenberger, *Cancer Research* **6**, 128 (1946).

TABLE IV

DESTHIOBIOTIN AND ITS ANALOGS[114]

Compound	Organism	Molar inhibition ratio			References
		Biotin	Oxybiotin	Desthiobiotin	
(+)-Desthiobiotin	*Lactobacillus casei*	9,000			109, 136, 137
dl-Desthiobiotin	*Lactobacillus casei*	17,000	1,200		65, 124, 138
dl-2-Oxo-4-imidazolidinevaleric acid	*Saccharomyces cerevisiae*	>37,000,000		1,340,000	109, 168
	Lactobacillus casei	52,000,000			109, 168
dl-2-Oxo-4-imidazolidinecaproic acid	*Saccharomyces cerevisiae*	850,000		3,000	109, 168, 169
	Lactobacillus casei	100,000			109, 168, 169
	Pneumococcus type II or III	ca. 200,000[a]			168
	Escherichia coli	[b]		ca. 100	170
dl-2-Oxo-4-imidazolidine-enanthic acid	*Saccharomyces cerevisiae*	1,700,000		100,000	168
	Lactobacillus casei	1,600,000			168
dl-2-Oxo-4-imidazolidinecaprylic acid	*Saccharomyces cerevisiae*	2,000,000		270,000	168
	Lactobacillus casei	10,000,000			168
dl-5-Methyl-2-oxo-4-imidazolidine-enanthic acid	*Saccharomyces cerevisiae*	>3,300,000			169, 171
	Lactobacillus casei	1,100,000			169
dl-5-Methyl-2-oxo-4-imidazolidine-pelargonic acid	*Saccharomyces cerevisiae*	200,000			65
	Lactobacillus casei	1,000,000			65
dl-5-Methyl-2-thiono-4-imidazolidinecaproic acid	*Lactobacillus casei*	450,000			142
dl-5-Methyl-2-oxo-4-imidazolidine-pentanesulfonic acid	*Saccharomyces cerevisiae*	300,000	17,000	40,000	172
	Lactobacillus casei	[c]			172
dl-4-Butyl-5-methyl-2-imidazolidine	*Saccharomyces cerevisiae*	1,300,000			169
	Lactobacillus casei	1,000,000			169
dl-4-Hexahydrobenzyl-5-methyl-2-imidazolidone	*Saccharomyces cerevisiae*	370,000			65
	Lactobacillus casei	[c]			65

[a] Inhibition index for complete inhibition.
[b] Non-competitive reversal of toxicity.
[c] Not inhibitory.

TABLE V[114]

HOMOLOGS AND SULFONE ANALOGS OF BIOTIN

Compound	Organism	Molar inhibition ratio Biotin	Desthiobiotin	References
dl-Norbiotin	*Saccharomyces cerevisiae*	1,000		174
	Lactobacillus casei	13,000		174
dl-Homobiotin	*Saccharomyces cerevisiae*	700		174
	Lactobacillus casei	130		174
	Lactobacillus arabinosus	43,000		174
dl-Bishomobiotin	*Saccharomyces cerevisiae*	30,000		174
	Lactobacillus casei	7,000		174
dl-Trishomobiotin	*Saccharomyces cerevisiae*	50,000		174
	Lactobacillus casei	3,000		174
Biotin sulfone[a]	*Lactobacillus casei*	280		109
	Neurospora crassa	ca. 1,000	ca. 1,000	143
	Escherichia coli 58[b]	ca. 1,000	ca. 1,000	143
dl-Homobiotin sulfone	*Saccharomyces cerevisiae*	40,000		174
	Lactobacillus casei	400		174
dl-Bishomobiotin sulfone	*Saccharomyces cerevisiae*	60,000		174
	Lactobacillus casei	8,000		174
dl-Trishomobiotin sulfone	*Saccharomyces cerevisiae*	60,000		174
	Lactobacillus casei	6,000		174

[a] Also inhibitory for *L. arabinosus* and *Staphylococcus aureus*.
[b] A mutant strain requiring biotin or desthiobiotin for growth.

TABLE VI[114]

ANALOGS OF OXYBIOTIN

Compound	Organism	Molar inhibition ratio Biotin	Oxybiotin	References
dl-Bisnoroxybiotin	*Saccharomyces cerevisiae*	>500,000	[a]	74
	Lactobacillus arabinosus	>500,000		74
dl-Noroxybiotin	*Saccharomyces cerevisiae*	>500,000	143,000	74
	Lactobacillus arabinosus	>500,000		74
dl-Homoöxybiotin	*Saccharomyces cerevisiae*	445,000	7,400	74, 175
	Lactobacillus arabinosus	>500,000	225,000	74
dl-Bishomoöxybiotin	*Saccharomyces cerevisia*	>500,000	30,000	74
	Lactobacillus arabinosus	>500,000		74
dl-Hexahydro-2-oxo-1H-furo-[3,4]imidazole-4-(4-butane-sulfonic acid) (oxybiotin sulfonic acid)	*Saccharomyces cerevisiae*	1,460,000	16,600	76, 77, 175
dl-Hexahydro-2-oxo-4-(4-benzylthiobutyl)-1H-furo[3,4]-imidazole	*Saccharomyces cerevisiae*	740,000	9,300	76 77, 175

[a] No significant inhibition.

of the yeast cells when given to the culture before the addition of biotin or oxybiotin, with inhibitory ratios of 3750 and 5000, respectively, for oxybiotin, and 375,000 and 600,000 for biotin. No inhibition is observed when

[174] M. W. Goldberg, L. H. Sternbach, S. Kaiser, S. D. Heineman, J. Scheiner, and S. H. Rubin, *Arch. Biochem.* **14**, 480 (1947).
[175] A. E. Axelrod, S. E. Purris, and K. Hofmann, *J. Biol. Chem.* **176**, 695 (1948).

the addition of the biotin precedes that of the inhibitors. This indicates that the inhibition must prevent the conversion of biotin to a functional form which is not antagonized by the analogs.[176]

g. Ureylenephenyl and Ureylenecyclohexylbutyric and Valeric Acids

A group of 2,3- and 3,4-ureylenecyclohexylbutyric and similar valeric acid analogs as well as ureylenephenyl compounds were synthesized.[176] Some of them showed potent antibiotin activity (Table VII). The resem-

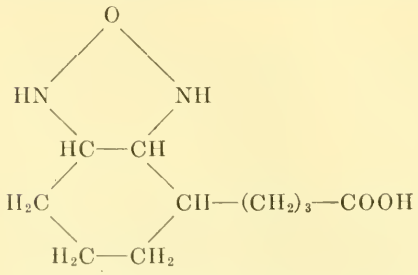
TABLE VII[114]

UREYLENEBENZENE AND CYCLOHEXANE DERIVATIVES AS INHIBITORY BIOTIN ANALOGS

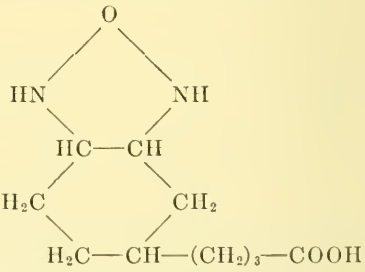
Analog	Molar inhibition rate	
	Lactobacillus casei	*Saccharomyces cerevisiae*
γ-(2,3-Ureylenephenyl)butyric acid	25,000,000	310,000
γ-(2,3-Ureylenecyclohexyl)butyric acid[a] m.p. 218–220°	12,500,000	1,500
γ-(2,3-Ureylenecyclohexyl)butyric acid[a] m.p. 192–194	6,250,000	1,500
δ-(2,3-Ureylenephenyl)valeric acid	6,250,000	2,500,000
δ-(2,3-Ureylenecyclohexyl)valeric acid,[a] m.p. 222–226°	31,000	3,000
δ-(2,3-Ureylenecyclohexyl)valeric acid,[a] m.p. 183–184°	31,000	3,000
γ-(3,4-Ureylenephenyl)butyric acid[b]	1,500,000	6,250,000
γ-(3,4-Urelenecyclohexyl)butyric acid[b]	4,000	156,000
δ-(3,4-Ureylenephenyl)valeric acid	750,000	1,560,000
δ-(3,4-Ureylenecyclohexyl)valeric acid	31,000	156,000

[a] Stereoisomeric modifications distintinguished only by melting point.
[b] Inhibits growth of *Lactobacillus arabinosus*.

blance of γ-(2,3-ureylenecyclohexyl)butyric acid and γ-(3,4-ureylenecyclohexyl)butyric acid to biotin is illustrated by their structure formulas.



γ-(2,3-Ureylenecyclohexyl)butyric acid



γ-(3,4-Ureylenecyclohexyl)butyric acid

[176] J. P. English, R. C. Clapp, Q. P. Cole, I. F. Halverstadt, J. O. Lampen, and R. O. Roblin, *J. Am. Chem. Soc.* **67**, 295 (1945).

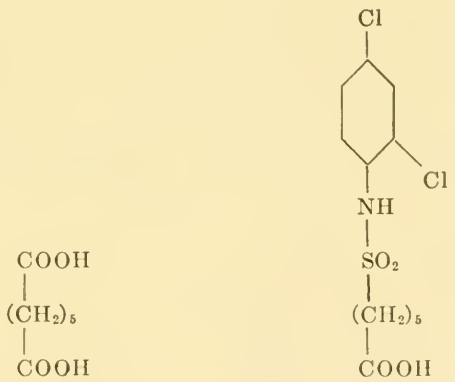
The two diastereoisomers tested have shown identical biological activity (Table VII).

h. Lysolecithin

Lysolecithin, which is free from unsaturated fatty acids, has a marked inhibitory effect on the utilization of biotin by *Lactobacillus casei*, with a molar inhibitory index of about 50,000 to 100,000. Oleic acid may counteract the inhibition caused by lysolecithin.[177] If biotin functions in the synthesis of oleic acid, lysolecithin might behave as a true competitive analog and block the synthesis of oleic acid. This might then explain the neutralizing effect of oleic acid on the inhibition caused by lysolecithin.

i. Analog of Pimelic Acid

ϵ-(2,4-Dichlorosulfanilido)caproic acid, an analog of pimelic acid, in which the dichlorosulfanilido group replaced one of the carboxyls of pimelic acid, has been shown to inhibit the growth of several bacteria which do not require biotin as a growth factor and not to affect those which do require biotin. Its harmful effect may be overcome competitively by pimelic acid as precursor of biotin and non-competitively by small amounts of biotin.[178]



Pimelic acid

ϵ-(2-4-Dichlorosulfanilido)-caproic acid

[177] W. Trager, *J. Bacteriol.* **56**, 195 (1948).
[178] D. W. Woolley, *J. Biol. Chem.* **183**, 495 (1950).

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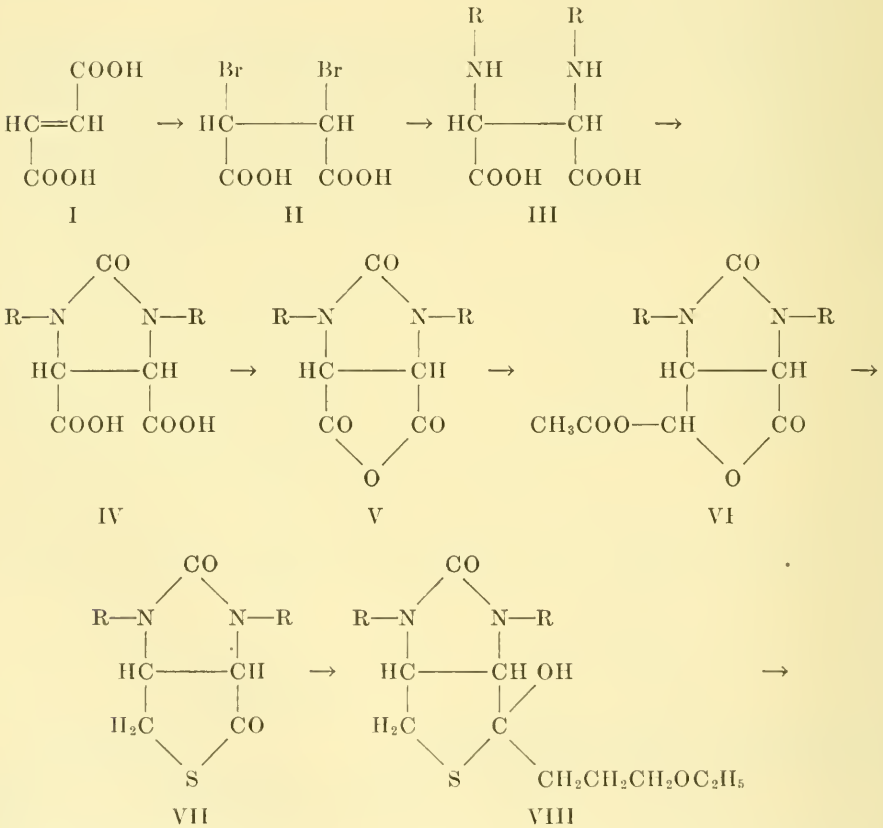
III. Industrial Preparation[1]

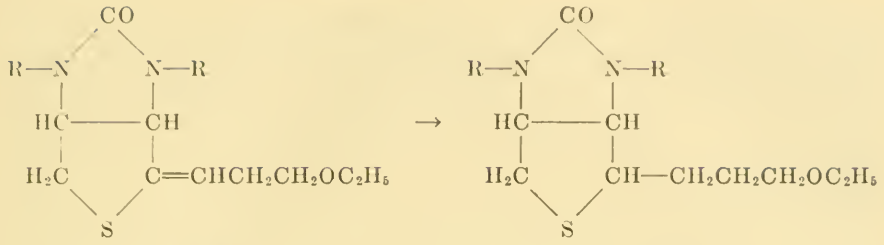
PAUL GYÖRGY

Biotin is being produced commercially by a synthesis developed in the Research Laboratories of Hoffman-LaRoche, Inc.[2] It differs entirely from the previously published syntheses and is characterized by the fact that a *meso*-diaminosuccinic acid derivative is being used as an intermediate. This automatically leads to the required *cis* structure of the final product, which is also obtained in optically active form, since the resolution into optical antipodes is being carried out at an intermediate stage. The following formulas describe the synthesis.

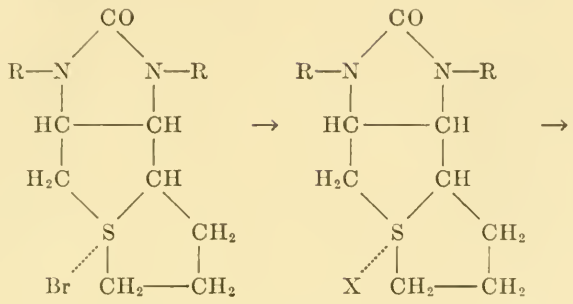


[1] The information contained in this section was kindly furnished by Dr. M. W. Goldberg of the Research Laboratories of Hoffmann-La Roche, Inc. Nutley, N. J.

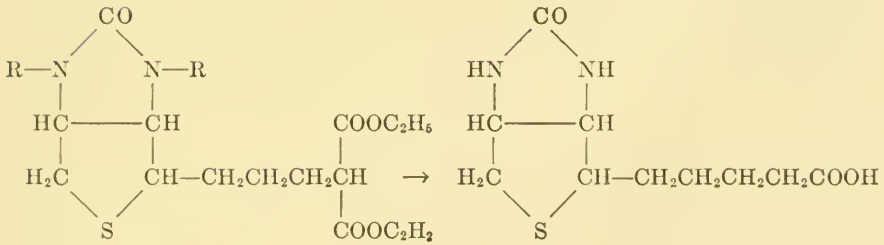
[2] M. W. Goldberg and L. H. Sternbach, U. S. Pats. 2,489,232–2,489,238, November 22, 1949.



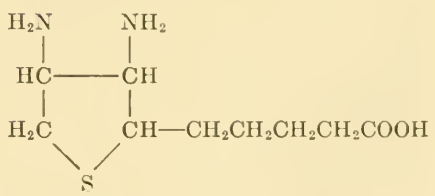
IX X



XI XII



XIII XIV



XV

R = Benzyl

X = *d*-Camphsulfonic acid ion

Starting material is fumaric acid (I), which is converted via *meso*-dibromosuccinic acid (II) to *meso*-α,β-bisbenzylaminosuccinic acid (III). The diamino acid is reacted with phosgene, giving 1,3-dibenzyl-2-imidazolidone-*cis*-4,5-dicarboxylic acid (IV). The anhydride (V) of the latter is then reduced with zinc in an acetic acid-acetic anhydride mixture forming the

acetate of the cyclic form of the corresponding aldehydo acid, 3,4-(1′,3′-dibenzyl-2′-ketoimidazolido)-2-keto-5-acetoxytetrahydrofuran (VI). Compound VI, when treated under suitable conditions with hydrogen sulfide in the presence of hydrogen chloride, followed by a reductive treatment of the intermediate formed, gives 3,4-(1′,3′-dibenzyl-2′-ketoimidazolido)-2-ketothiophane (VII).

The thiolactone (VII) can be converted to biotin by several routes. In the preferred method, it is reacted with 3-ethoxypropyl magnesium bromide to give 3,4-(1′,3′-dibenzyl-2′-ketoimidazolido)-2-hydroxy-2-(ω-ethoxypropyl)thiophane (VIII). The latter is dehydrated to the corresponding unsaturated compound IX, which is then hydrogenated to 3,4-(1′,3′-dibenzyl-2′-ketoimidazolido)-2-(ω-ethoxypropyl)thiophane (X), using Raney nickel as a catalyst. When compound X is treated with hydrogen bromide in acetic acid, it forms 3,4-(1′,3′-dibenzyl-2′-ketoimidazolido)-1,2-trimethylenethiophanium bromide XI. The thiophanium bromide is then converted to the corresponding *d*-camphorsulfonic acid salt by treatment with silver *d*-camphorsulfonate. This *d*-camphorsulfonate represents a mixture of two diastereomeric salts which can be readily separated. Crystallization from isopropanol gives the *l*-3,4-(1′,3′-dibenzyl-2′-ketoimidazolido-1,2-trimethylenethiophanium *d*-camphorsulfonate (XII), the optical configuration of which corresponds to that of the natural *d*-biotin. When this *l*-thiophanium *d*-camphorsulfonate is treated with sodium diethylmalonate in toluene, it forms a *levo*-rotatory 3,4-(1′,3′-dibenzyl-2′-ketoimidazolido)-2-(ω,ω-dicarbethoxybutyl)thiophane (XIII). This product can be converted to *d*-biotin (XIV) in a one-step reaction by simply boiling it with concentrated hydrobromic acid. This treatment causes saponification of the two ester groups present in the side chain and decarboxylation of the corresponding free malonic acid, to form the ω-carboxybutyl side chain present in biotin. Simultaneously, the two benzyl groups which protect the nitrogen atoms of the imidazolidone ring throughout the synthesis are removed in form of benzyl bromide. In spite of the drastic conditions employed in this reaction the yield is surprisingly good, and only a small part of the biotin formed is hydrolyzed to the corresponding diamino acid (XV). The latter can be readily reconverted to *d*-biotin by treatment with phosgene.

The synthesis is highly stereospecific. No epibiotin is formed, and since the required *cis* configuration is present from the beginning, the two *trans* isomers, allobiotin and epiallobiotin, are also absent.

IV. Biochemical Systems

PAUL GYÖRGY

A. ENZYMES AND COENZYMES

Since the first demonstration of the double role of riboflavin as a vitamin and a "proenzyme,"[1-3] the participation of the various B vitamins in enzyme systems has been clearly demonstrated and for each newly discovered member of this vitamin group generally anticipated as a matter of statistical certainty. This approach has also been followed for biotin. The search for coenzymes or other enzymatic compounds in which biotin should play a determining role has been pursued in various directions. It appears probable that biotin, by analogy to riboflavin or other members of the B vitamin group, acts in biochemical systems as part of the coenzymes, and not in free form but bound on protein or similar higher molecular compounds. If "flavoprotein" is the enzymatically active form of riboflavin,[3] then "biotoprotein"[4] may represent its equivalent for biotin. The fact that biotin occurs in tissues, chiefly in bound form (p. 529), is in good accord with this view. In spite of various attempts, the isolation and identification of biotin-containing coenzymes has not yet been achieved[5] or at least not fully accomplished.[6-8] Even for biocytin it may be stated that it is not the true coenzyme form of biotin but probably only a constituent of a higher molecular biotin compound.[9, 10]

B. MECHANISM OF ACTION

A number of enzymatic reactions have been discovered and analyzed in which biotin appears to participate in a direct or indirect manner. The best and most conclusive results were obtained by the study of the microbiological system or of the metabolism of tissues obtained from biotin-deficient animals and finally by the study of isolated enzyme systems. The evidence so far accumulated indicates that biotin seems to function in several enzymatic processes of which the common denominator is not easily determined and which may involve several, perhaps independent,

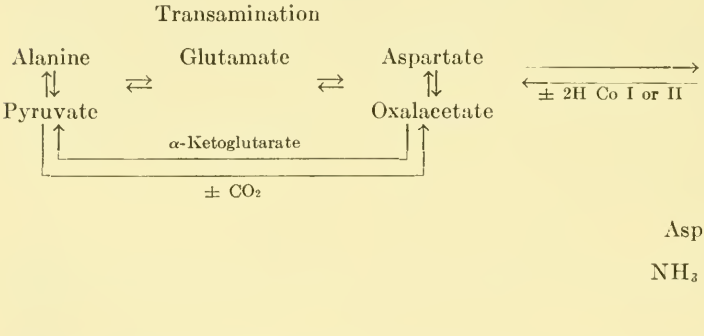
[1] P. György, R. Kuhn, and T. Wagner-Jauregg, *Naturwissenschaften* **21**, 560 (1933).
[2] R. Kuhn, P. György, and T. Wagner-Jauregg, *Ber.* **66**, 317 (1933).
[3] P. György, R. Kuhn, and T. Wagner-Jauregg, *Hoppe-Seyler's Z. physiol. Chem.* **223**, 241 (1934).
[4] K. Hofmann, D. F. Dickel, and A. E. Axelrod, *J. Biol. Chem.* **183**, 481 (1950).
[5] D. R. Miller, J. O. Lampen, and W. H. Peterson, *J. Am. Chem. Soc.* **65**, 2369 (1943).
[6] H. C. Lichtstein and J. F. Christman, *J. Bacteriol.* **58**, 565 (1949).
[7] J. F. Christman and H. C. Lichtstein, *J. Bacteriol.* **60**, 107 (1950).
[8] H. C. Lichtstein, *J. Bacteriol.* **60**, 485 (1950).
[9] H. C. Lichtstein, J. F. Christman, and W. L. Boyd, *J. Bacteriol.* **59**, **113** (1950).
[10] G. Feldott, P. R. MacLeod, and H. A. Lardy, *Federation Proc.* **9**, 170 (1950).

metabolic functions. The first suggestion regarding the enzymatic action of biotin related it to the synthesis of aspartic acid[11-13] and to the oxidation of pyruvic acid,[14] the latter being probably the result of faulty carbon dioxide transfer in the absence of biotin.[15] As further major functions the deamination of certain amino acids and the biosynthesis of oleic acid, and as minor functions the relationships to carbohydrate synthesis, succinic acid dehydrogenase, and amino acid oxidase, should be mentioned.

1. Biotin in Aspartic Acid Synthesis

The sparing effect of aspartic acid on the biotin requirement of yeast and the growth promotion of yeast by aspartic acid in partial replacement of biotin[11-13] has been extended to various species of bacteria.[16-20]

The cellular synthesis of aspartic acid may involve three possible reactions: (1) transamination, (2) the condensation of pyruvate and carbon dioxide to yield oxalacetate (Wood-Werkman reaction[21]), and (3) the direct amination of fumaric acid, as illustrated by the following scheme.



The fact that resting cells of *Lactobacillus arabinosus, Streptococcus faecalis R.*, and *L. casei* are able to form aspartic acid from glutamic acid, alanine, or cysteic acid plus oxalacetic acid at the same rate with or with-

[11] D. Burk, R. J. Winzler, and V. du Vigneaud, *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.* **140,** XXI (1941).
[12] S. A. Koser, M. H. Wright, and A. Dorfman, *Proc. Soc. Exptl. Biol. Med.* **51,** 204 (1942).
[13] R. J. Winzler, D. Burk, and V. du Vigneaud, *Arch. Biochem.* **5,** 25 (1944).
[14] F. J. Pilgrim, A. E. Axelrod, and C. A. Elvehjem, *J. Biol. Chem.* **145,** 237 (1942).
[15] D. Burk and R. J. Winzler, *Science* **97,** 57 (1943).
[16] J. L. Stokes, A. Larsen, and M. Gunness, *J. Biol. Chem.* **167,** 613 (1947).
[17] J. L. Stokes, A. Larsen, and M. Gunness, *J. Bacteriol.* **54,** 219 (1947).
[18] H. A. Lardy, R. L. Potter, and C. A. Elvehjem, *J. Biol. Chem.* **169,** 451 (1947).
[19] W. Shive and L. J. Rogers, *J. Biol. Chem.* **169,** 453 (1947).
[20] R. L. Potter and C. A. Elvehjem, *J. Biol. Chem.* **172,** 531 (1948).
[21] C. H. Werkman and H. C. Wood, *Botan. Rev.* **8,** 1 (1942).

out biotin eliminates biotin as an essential factor in the transamination reaction.[17] Although no direct evidence was found for the direct amination of fumaric acid in *L. arabinosus*,[17] the observation that biotin-deficient yeast cells are markedly stimulated by ammonia[13] does not exclude the possibility that biotin may be concerned in aspartic acid deaminase.

More direct evidence is available for the role of biotin in the Wood-Werkman reaction. Resting cell suspensions of *L. arabinosus* supplied with glutamate[17] or any C_4-decarboxylic acid[22] do not in the absence of biotin produce aspartate upon the addition of pyruvate, or pyruvate plus $NaHCO_3$ or carbon dioxide. On the other hand, oxalacetate may partially replace biotin in aspartic acid-deficient media for *L. casei* and *L. arabinosus*,[18] but not for *S. faecalis R*. Bicarbonate greatly stimulates growth of *L. arabinosus* in aspartate-free media if biotin is present but is without any effect in media low in biotin content.[18]

Although all these observations as well as "inhibition analysis"[19] seem to support the pertinent data that biotin may participate in carbon dioxide fixation into oxalacetate and α-ketoglutarate, objections nevertheless may be raised to the conclusion that the effect of biotin on aspartic acid synthesis should pass exclusively through the Wood-Werkman reaction. The following observations should be mentioned in favor of some other, at least supplementary, mechanism: (a) aspartic acid is always more effective than oxalacetic acid in substituting for biotin; (b) other C_4-decarboxylic acids, which should be easily converted to oxalacetic acid, are inactive both as substitutes for biotin and as agents for reversing biotin inhibitors.[22]

2. Biotin in Carbon Dioxide Fixation and Decarboxylation

Studies of the biotin-asparate interrelationship furnished sufficient proof for the assumption that biotin, or rather a biotin coenzyme, is concerned with the condensation of carbon dioxide and pyruvate to form oxalacetate and aspartate or in reversed direction with the decarboxylation of oxalacetic acid.[17-19, 23]

$$\text{Oxalacetic acid} \rightleftarrows \text{Pyruvic acid} + CO_2$$

In a similar manner biotin may be involved in the decarboxylation of oxalosuccinic acid.[19]

$$\text{Oxalosuccinic acid} \rightleftarrows \alpha\text{-Ketoglutaric acid} + CO_2$$

In *E. coli* α-ketoglutaric acid effected a threefold increase in the antibacterial index for the competitive inhibition of biotin by desthiobiotin

[22] R. J. Williams, R. E. Eakin, E. Beerstecher, Jr., and W. Shive, The Biochemistry of B Vitamins. Reinhold Publishing Corp., New York, 1950.

[23] H. C. Lichtstein and W. W. Umbreit, *J. Biol. Chem.* **170**, 329 (1947).

This indicates that inhibition of the growth effect of biotin interferes with the biosynthesis of α-ketoglutaric acid, formed under usual circumstances by decarboxylation of oxalosuccinic acid.[19]

In a similar example of "inhibition analysis"[19] in *L. arabinosus*, the antibacterial index for competitive inhibition of biotin by γ-(3,4-ureylenecyclohexyl)butyric acid was increased tenfold by the addition of aspartic acid or oxalacetic acid. Here apparently biotin regulates the synthesis of oxalacetic acid.

It has been further shown[23] that *E. coli* harvested from a complex medium, on standing in molar phosphate at pH 4, rapidly lost the ability to produce carbon dioxide from aspartic acid as well as from malic acid or

TABLE VIII[a]

Age, days	Diet	"Malic" enzyme		Biotin per gram of acetone powder, γ
		TPN reduction	Oxalacetate decarboxylation	
30	Normal (3)[b]	2.20	2.40	3.70
	No biotin (6)	0.96	1.15	0.78
44	Normal (1)	1.83	3.36	4.50
	No biotin (1)	0.75	1.79	
	Low biotin (2)	0.64	1.90	0.68
	Low folic (2)	1.50	2.83	5.65
50	Normal (3)	1.59	2.89	6.15
	Low biotin (2)	0.91	1.83	0.41
	Low folic (1)	2.34	3.79	4.28

[a] Adapted from Ochoa *et al.*[24] by Lichtstein.[25]

[b] Figures in parentheses equal number of livers pooled in each case.

oxalacetic acid. Addition of biotin restored this lost enzymatic function. Although the metabolic product derived from malate in *E. coli* appears to be different from oxalacetate, and there is no proof that under similar conditions aspartic acid would be converted by *E. coli* to oxalacetic acid,[23] the fact that *E. coli* requires biotin to produce carbon dioxide from these compounds indicates its functional role in decarboxylation.

The decarboxylation of oxalacetate or the ability of malate to reduce triphosphopyridine nucleotide has been found to be reduced in the livers obtained from biotin-deficient turkeys.[24] Several other dehydrogenases tested were not altered, and the effect of biotin appeared to be specific (Table VIII). However, biotin restored the normal enzyme content only

[24] S. Ochoa, A. Mehler, M. S. Blanchard, T. H. Jukes, C. E. Hoffmann, and M. Regan, *J. Biol. Chem.* **170**, 413 (1947).

[25] H. C. Lichtstein, *Vitamins and Hormones* **9**, 27 (1951).

when given to the living animal. Reactivation of the "malic" enzyme was not achieved in vitro by biotin or by boiled extracts of either normal liver or purified pigeon liver enzyme. It was further found that purified preparations of the pigeon liver "malic" enzyme were essentially devoid of biotin.[24] The suggestion was made that either biotin occurs in "malic" enzyme in a microbiologically inactive form or its relation to the enzymatic reaction in question is of a less direct nature than of a prosthetic group in carbon dioxide fixation enzyme systems.

More recently it has been shown[26] that the "malic" enzyme may be enriched in an adaptive manner in *Lactobacillus arabinosus* by culturing the organism in the presence of malic acid. In the absence of biotin in the medium even with malate present, the increase of "malic" enzyme is greatly retarded and reduced, in comparison to the concentration of the enzyme in cultures grown in the presence of malate *and* biotin. Addition of biotin alone will not increase the activity of "malic" enzyme. Biotin and a small amount of the growth medium or its component amino acids together with glucose, furnishing glycolytic energy, will slowly and partially reactivate the malate dissimilation system of biotin-deficient organisms.[26]

$$l\text{-Malate} = \text{Lactate} + CO_2$$

These[26] and previous[24] studies suggest that "the relationship of biotin to enzyme systems of carbon dioxide fixation is less direct than that of a prosthetic group or a component of a prosthetic group. It appears that biotin may be involved in some manner in the synthesis of these and possibly of other enzymes."[26]

Observations on the deranged pyruvate and succinate metabolism in cardiac muscle slices from biotin-deficient ducks are consistent with the latter view.[27] $C^{14}O_2$ production from carboxyl-labeled succinate was greatly reduced in such muscle slices. Addition of biotin *in vitro* was without effect, and intraperitoneal administration of biotin to deficient ducks was required to restore normal enzymatic function.[27]

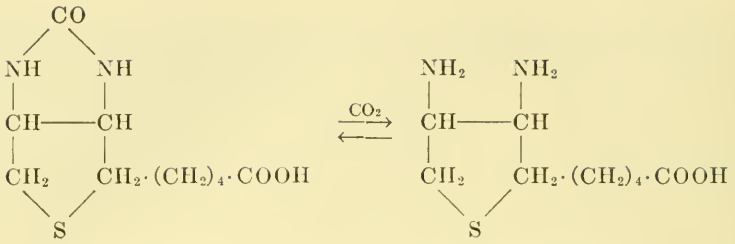
It was early postulated[15] that biotin might enter into biological carbon dioxide-transferring mechanisms by virtue of opening and closing of the ureido ring system.

Biotin labeled with C^{14} in the ureido carbon atom has been synthesized[28] by the diaminocarboxylic acid with radioactive phosgene. In cultures of *Lactobacillus arabinosus* with the addition of such tagged biotin under conditions requiring its participation in carbon dioxide fixation no replacement

[26] M. L. Blanchard, S. Korkes, A. del Campillo, and S. Ochoa, *J. Biol. Chem.* **187**, 875 (1950).

[27] R. E. Olson, O. N. Miller, Y. J. Topper, and F. J. Stare, *J. Biol. Chem.* **175**, 503 (1948).

[28] D. B. Melville, J. G. Pierce, and C. W. H. Partridge, *J. Biol. Chem.* **180**, 299 (1949).



of C^{14} by C^{12} was observed. Thus, the ureido carbonyl group is apparently not transferred during carbon dioxide fixation.[28]

Fixation of carbon dioxide in mammalian tissues and in microorganisms under the influence of biotin has been clearly demonstrated.[29-31] In the presence of biotin, *L. arabinosus* fixed C^{14} from bicarbonate into cellular aspartic acid. However, no fixation was observed when the medium contained less than 0.05 mγ biotin per milliliter or when aspartic acid or an antibiotin was added. Biotin analogs having antimetabolite properties inhibited the fixation of carbon dioxide when added simultaneously with biotin to cells grown on a low-biotin medium. They had little influence on the carbon dioxide fixing capacity of cells grown in the presence of biotin.[29]

Oxybiotin is inherently less effective than biotin for carbon dioxide fixation even at neutral pH.[32]

Intraperitoneal injection of $NaHCO_3$ containing C^{14} into normal and biotin-deficient rats resulted in a larger C^{14} fixation into adenine, guanine, arginine, aspartic acid, citric acid, and bone carbonate of the control animals than into the corresponding compounds of the biotin-deficient animals. The presence of C^{14} in bone citrate indicates that this citrate is in dynamic equilibrium with other tissue citrate. "The small fixation is probably due to the fact that the major portion of the isotopic citrate formed would be immediately oxidized via the citric acid cycle."[30]

The biotin-deficient rat exhibits lower carbon dioxide fixation into tissue arginine than normal controls. The Krebs-Henseleit cycle[33] involves urea formation through the intermediate of arginine, and this again passes through the overall step of carbon dioxide fixation via ornithine → citrulline.[34] The synthesis of citrulline from ornithine is significantly decreased in the liver homogenates of biotin-deficient rats. The impaired synthesis by the deficient liver may be increased by the addition *in vitro* of a heated residue

[29] H. A. Lardy, R. L. Potter, and R. H. Burris, *J. Biol. Chem.* **179**, 721 (1949).
[30] P. R. MacLeod and H. A. Lardy, *J. Biol. Chem.* **179**, 733 (1949).
[31] P. R. MacLeod, S. Grisolia, P. P. Cohen, and H. A. Lardy, *J. Biol. Chem.* **180**, 1003 (1949).
[32] R. L. Potter and C. A. Elvehjem, *J. Biol. Chem.* **183**, 587 (1950).
[33] H. A. Krebs and K. Henseleit, *Hoppe-Seyler's Z. physiol. Chem.* **210**, 33 (1932).
[34] S. Grisolia and P. P. Cohen, *J. Biol. Chem.* **176**, 929 (1948).

of normal rat liver homogenate or within 24 hours after injection of 200 γ of biotin into deficient animals.[31]

The synthesis of citrulline from ornithine, glutamate, carbon dioxide, and ammonia by washed residue of rat liver homogenate[35, 36] is greatly reduced when the liver enzyme preparation is obtained from biotin-deficient rats.[31] Inasmuch as it is carbamyl-L-glutamate rather than glutamate which acts as actual catalytic intermediate in citrulline synthesis from ornithine,[37, 38] it would be interesting to learn whether the effect of biotin on citrulline synthesis may be placed prior to or subsequent to the reactions in which carbamyl-L-glutamate participates. It has been shown by means of enzymatic preparations obtained from normal and biotin-deficient rats that replacement of glutamate by carbamyl-L-glutamate has compensated for the lack of the specific "biotin-enzyme preparation" and resulted in equal rates of citrulline synthesis by the biotin-deficient and control preparations.[39] Futhermore, inhibition of citrulline formation by the biotin-deficient liver enzyme preparation in the presence of glutamate was affected by fumarate, oxalacetate, and aspartate. These observations[39] are in good accord with the assumption that "the influence of biotin on CO_2 fixation into citrulline is at a step prior to that at which carbamyl-L-glutamate functions in the conversion of ornithine to citrulline.[39]

The incorporation of $C^{14}O_2$ from bicarbonate into the carboxyl group of acetoacetate formed from crotonate, butyrate, capronate, caprylate, isovalerate, heptylate, nonylate, or pyruvate by rat liver homogenates[40] is markedly decreased when the enzyme is prepared from biotin-deficient animals.[41] However, the quantity of acetoacetate as such is not affected by the state of biotin nutrition of the animals. The incorporation of $C^{14}O_2$ into the carboxyl group of acetoacetate metabolically formed from pyruvate, caproate, caprylate, or isovalerate was increased ten- to one hundred-fold when biotin-deficient rats received biotin either in their diet or by injection.[42] These observations offer further proof for the important role of biotin in the intermediate carbon dioxide fixation mechanism.

In a cell-free enzyme system prepared from *Micrococcus lysodeikticus* the addition of avidin will prevent the fixation of carbon dioxide in oxalacetate. The addition of biotin in excess results in a return of normal fixation.[43]

[35] P. P. Cohen and M. Hayano, *J. Biol. Chem.* **170**, 687 (1947).
[36] P. P. Cohen and M. Hayano, *J. Biol. Chem.* **172**, 405 (1948).
[37] P. P. Cohen and S. Grisolia, *J. Biol. Chem.* **182**, 747 (1950).
[38] S. Grisolia and P. P. Cohen, *J. Biol. Chem.* **191**, 189 (1951).
[39] G. Feldott and H. A. Lardy, *J. Biol. Chem.* **192**, 447 (1951).
[40] G. W. E. Plaut and H. A. Lardy, *J. Biol. Chem.* **192**, 435 (1951).
[41] G. W. E. Plaut and H. A. Lardy, *J. Biol. Chem.* **186**, 705 (1950).
[42] G. W. E. Plaut, *Proc. Soc. Exptl. Biol. Med.* **78**, 769 (1951).
[43] G. E. Wessman and C. H. Werkman, *Arch. Biochem.* **26**, 214 (1950).

3. Biotin in Deamination

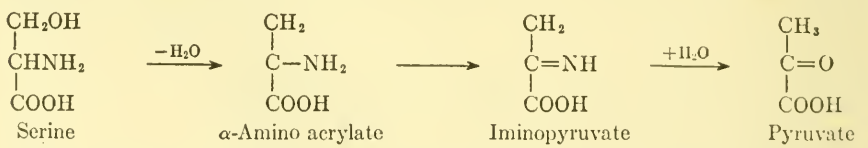
The possibility that deamination may play a part in the metabolic interrelation of aspartic acid and oxalacetic acid[13] has been already mentioned (p. 576), and it has been made further probable by the finding that in aging bacterial cells the deamination of aspartic acid is markedly impaired and may be restored by the addition of biotin. Similar observations were made on the deamination of serine and threonine.[44] Table IX contains some of the pertinent data.[25, 44]

TABLE IX

Biotin Activation of Deaminases

Organism	Substrate	pH	Q NH_3/N		
			No addition	Vitamins less biotin	Biotin, 0.1 γ/ml.
Escherichia coli (Gratia)	L-Aspartic	4	96	108	304
	acid	4	27	29	102
		5	220	241	571
		7	419	467	752
P. vulgaris		7	100	121	377
Escherichia coli (86g)		7	154	154	279
Bacterium cadaveris		7	76	84	238
Escherichia coli (Gratia)	L-Serine	7	89		159
P. vulgaris		7	273	270	488
Escherichia coli (86g)		7	258	260	403
Bacterium cadaveris		7	117	117	249
Escherichia coli (86g)	DL-Threonine	7	52		122
		7	178	175	290
		7	29		78
Bacterium cadaveris		7	45	56	161

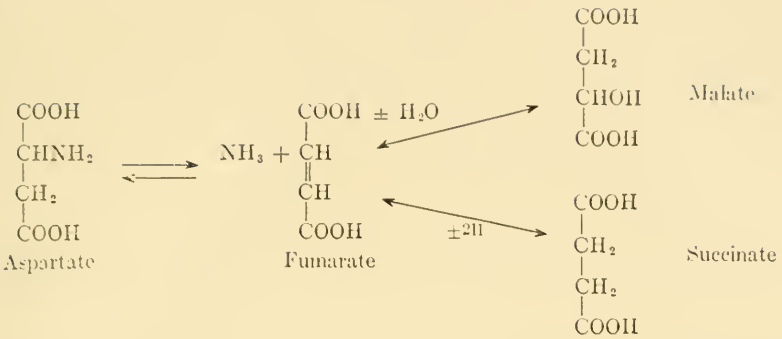
In the deamination of serine or threonine, first water is removed with the formation of the imino acid, leading in turn to the corresponding keto acid, in the case of serine to pyruvic acid.[25] The reaction is not reversible.



Serine — α-Amino acrylate — Iminopyruvate — Pyruvate

The deamination of aspartic acid may proceed through fumarate to malate or to succinate according to the following scheme.

[44] H. C. Lichtstein and W. W. Umbreit, *J. Biol. Chem.* **170**, 423 (1947).



In this fully reversible deamination no keto analog is produced; neither oxalacetate nor its decomposition product, pyruvate.

The deamination of alanine, phenylalanine, methionine, or glutamic acid was not stimulated by biotin under experimental conditions under which biotin was found to be effective in promoting the deamination of aspartic acid.

In aging cells the deaminase activity may be restored not only by biotin but also by muscle adenylic acid (adenosine-5-phosphoric acid). Furthermore it has been found that both biotin and adenylic acid stimulate the formation of aspartic acid from ammonia and malate, probably owing to a direct action on the deaminase rather than to a removal of oxalacetate from the reaction system.[45] The stimulation by biotin is almost immediate whereas that by adenylic acid usually requires a lag period. With prolonged incubation the stimulation by adenylic acid may exceed that produced by biotin. "This was taken as an indication that adenylic acid may be serving as a non-specific energy source supplying the energy necessary to synthesize the active coenzyme form of biotin, or that it is specifically necessary to phosphorylate biotin."[25]

In some experiments the reactivation of "aging cells" was achieved by biotin only at pH 7.0 but not at pH 4.[46] In other instances it was found[47] that during aging the cells may become more dependent on adenylic acid and will not respond to biotin. With the change in the composition of the media, "aging" may produce biotin deficiency or adenylic acid deficiency.[48]

Even in cell-free juice prepared from *E. coli*, dialyzed against phosphate

[45] H. C. Lichtstein and J. F. Christman, *J. Biol. Chem.* **175**, 649 (1948).

[46] L. D. Wright, E. L. Cresson and H. R. Skeggs, *Proc. Soc. Exptl. Biol. Med.* **72**, 556 (1949).

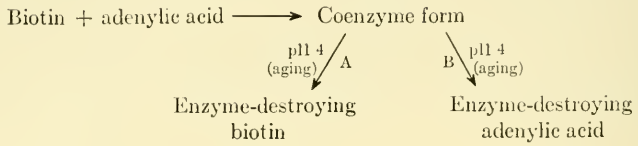
[47] A. E. Axelrod, K. Hofmann, S. E. Purvis, and M. Mayhall, *J. Biol. Chem.* **175**, 991 (1948).

[48] H. C. Lichtstein, *J. Biol. Chem.* **177**, 487 (1949).

[49] S. J. Ajl, W. R. Hart, and C. H. Werkman, *Enzymologia* **14**, 1 (1950).

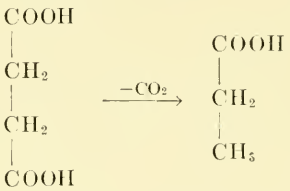
buffer and dilute KCl solutions, addition of biotin increases the deamination of aspartic acid.[49]

The interdependence of biotin and adenylic acid during "aging" of bacterial cells may be expressed by the following hypothetical scheme.[25]



4. Biotin in Other Enzyme Systems

The decarboxylation of succinic acid to propionic acid appears to require the presence of biotin.[50, 51] In a biotin-deficient medium the production



of carbon dioxide from added succinate is reduced and may be enhanced by biotin.

The metabolism of succinic acid is further linked with biotin through the succinic acid dehydrogenase. The activity of this enzyme is appreciably impaired in a cell-free extract of *E. coli* which has been dialyzed against phosphate buffer and dilute KCl solution. Full activity is restored by the addition of biotin.[49]

Biotin may also be related to amino acid oxidases, and thus in further consequence perhaps to protein synthesis. Both D- and L-amino acid oxidases are formed by suitable strains of *Neurospora crassa* in a synthetic medium containing 0.25 γ of biotin per liter. By increasing the concentration of biotin to 5 γ per liter the production of L-amino acid oxidase is suppressed and that of D-amino acid oxidase is maintained.[52]

The difficult enzymatic interrelationship between biotin and oleic acid has already been discussed (p. 564). Its detailed mechanism has not yet been elucidated.

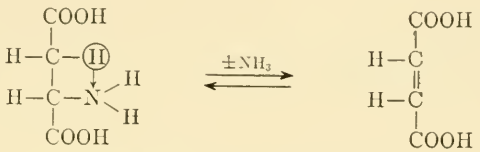
[50] E. A. Delwiche, *J. Bacteriol.* **59**, 439 (1950).
[51] H. C. Lichtstein, *J. Bacteriol.* **60**, 485 (1950).
[52] A. E. Bender, H. A. Krebs, and N. H. Horowitz, *Biochem. J.* **45**, xxi (1949).

5. Summary

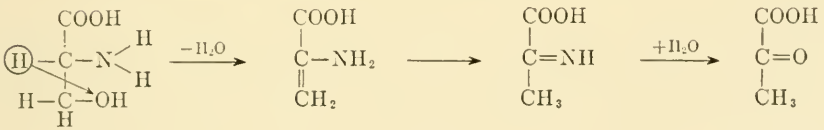
Biotin appears to participate in various enzymatic reactions such as in the decarboxylation of oxalacetate and succinate, in the deamination of aspartic acid, serine, and threonine, in the dehydrogenation of succinic acid, and indirectly in the synthesis of citrulline and oleic acid.

The theoretical attempt has been made to explain practically all these enzymatic reactions by one single mechanism, namely by intra- or intermolecular hydrogen transport.[25] The following schemes were devised for the various specific enzyme reactions:

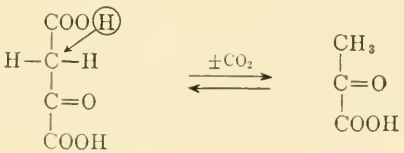
a. Aspartic Acid Deaminase



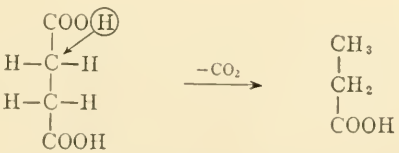
b. Serine and Threonine Deaminase (Illustrated for Serine)



c. Oxalacetate Decarboxylase

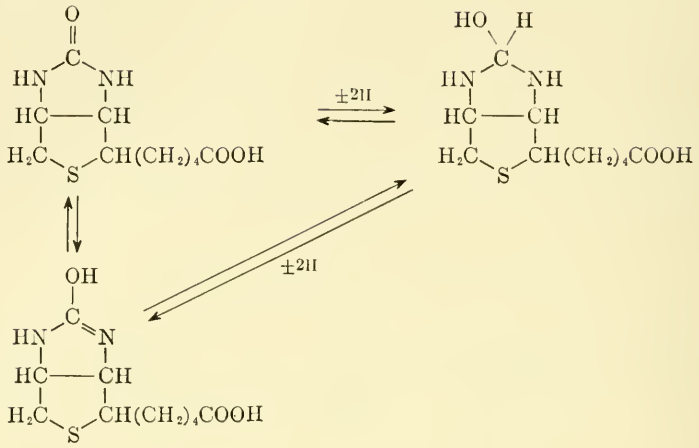


d. Succinate Decarboxylase



The role of biotin in this hydrogen transport may be summarized by

the following set of reactions, which should be amenable to experimental verification.[25]



V. Specificity of Action

PAUL GYÖRGY

Although there is probably only one form of biotin, a large number of analogs or isomers are known with some biological activity for animals and especially for microorganisms. They were discussed and tabulated in the section dealing with the chemical specificity of biotin and its analogs (p. 558ff). Oleic acid, pimelic acid, and similar compounds, although chemically not related to biotin, may spare biotin in some lower forms of life.

The best way of producing biotin deficiency in animals is the addition of avidin to the diet. Avidin binds biotin in the intestinal tract and by preventing the absorption of biotin causes a progressive deficiency state. This deficiency state may be averted or cured by the addition of biotin in excess to the diet or by the parenteral administration of biotin or its biologically active analogs.

Microorganisms possess a definite configurational specificity with regard to biotin activity in addition to responding differently to various analogs of biotin and its precursors. In general the *dextro*-rotatory form of biotin and its analogs is the biologically active steroisomer. *Leuconostoc dextranicum* elai is unique in that *dl* forms of oxybiotin and desthiobiotin are equal to *d*-biotin in allowing maximum growth in aspartate-free fructose medium. In the absence of any demonstrable time lag it may be assumed[1]

[1] V. Whiteside-Carlson, W. R. Starnes, C. L. Rosano, and W. W. Carlson, *Proc. Soc. Exptl. Biol. Med.* **77**, 344 (1951).

that these biotin analogs are utilized directly without prior conversion to the vitamin.

VI. Biogenesis

PAUL GYÖRGY

The best sources of biotin are yeast,[1, 2] lower fungi including *Aspergilli* and *Penicillia*,[3] and bacteria.[4] It may be assumed that animals derive the biotin they require, at least in part, from such microbiological synthesis of biotin. The amount of biotin released by bacteria into the culture medium may be several times that present in bacterial cells.[5] It is not due to the autolysis of dead cells, and it appears to be the vital function of the living microorganisms.

It has been shown that bacteria in the rumen of cattle are biotin producers.[6, 7] The fact that rats and man on a diet low in biotin excrete much more biotin than they do when biotin is supplied in the diet is also explained by the synthesis of biotin inside the body, through activity of the intestinal bacteria. In man the urinary output of biotin is roughly proportional to the intake, whereas the fecal excretion may greatly exceed the intake.[8-12] In rats receiving raw egg white in their diet the amount of biotin excreted in the urine and in the feces far exceeds that in the diet. This excess biotin must have originated chiefly from the non-dietary source of intestinal synthesis.[13]

[1] R. J. Williams, R. E. Eakin, and E. E. Snell, *J. Am. Chem. Soc.* **62**, 1204 (1940).
[2] L. H. Leonian and V. G. Lilly, *Science* **95**, 658 (1942).
[3] B. C. J. G. Knight, *Vitamins and Hormones* **3**, 105 (1945).
[4] W. H. Peterson and M. S. Peterson, *Bacteriol. Revs.* **9**, 49 (1945).
[5] R. C. Thompson, *Univ. Texas Publ.* **4237**, 87 (1942).
[6] L. W. McElroy and T. H. Jukes, *Proc. Soc. Exptl. Biol. Med.* **45**, 296 (1940).
[7] M. I. Wegner, A. N. Booth, C. A. Elvehjem, and E. B. Hart, *Proc. Soc. Exptl. Biol. Med.* **45**, 769 (1940).
[8] T. W. Oppel, *Am. J. Med. Sci.* **204**, 886 (1942).
[9] J. Gardner, H. T. Parsons, and W. H. Peterson, *Arch. Biochem.* **8**, 339 (1945).
[10] J. Gardner, H. T. Parsons, and W. H. Peterson, *Am. J. Med. Sci.* **211**, 198 (1946).
[11] C. W. Denko, W. E. Grundy, J. W. Porter, G. H. Berryman, T. E. Friedemann, and J. B. Youmans, *Arch. Biochem.* **10**, 33 (1946).
[12] C. W. Denko, W. E. Grundy, N. C. Wheller, C. R. Henderson, G. H. Berryman, T. E. Friedemann, and J. B. Youmans, *Arch. Biochem.* **11**, 109 (1946).
[13] M. A. McGregor, H. R. Parsons, and W. H. Peterson, *J. Nutrition* **33**, 517 (1947).

Rats in which coprophagy has been prevented grew better when biotin (and folic acid) were added to the diet, whereas in control animals with access to their own feces a dietary requirement for supplementary biotin (and folic acid) was not demonstrable.[14] Finally, the addition of sulfa drugs, such as succinylsulfathiazole, to rats kept on a purified semisynthetic diet not only retarded growth but also produced symptoms strongly reminiscent of egg white injury.[15-20] These latter manifestations may be relieved by adding biotin to the diet. The biotin content of the liver is decreased in rats when succinylsulfathiazole is added to the diet.[21] All these observations are in good accord with the assumption that biotin is synthesized by the intestinal flora.

Dietary carbohydrates may influence the fecal flora and indirectly the intestinal synthesis of biotin.[22, 23] This is promoted in chicks by added dextrin but not by sucrose or lactose. Oats have an especially marked effect on the bacterial synthesis of biotin.[24]

If green plants are able to synthesize biotin, the synthesis must take place in the leaves rather than in the roots. Root production is stimulated by biotin in etiolated cuttings of pea roots,[25] and under ordinary conditions of plant growth the roots may depend for at least part of their biotin supply on soil microorganisms.

VII. Estimation

PAUL GYÖRGY

In natural food products and even in concentrates, biotin is present only in extremely small concentrations. Consequently its estimation depends

[14] V. H. Barki, P. H. Derse, R. A. Collins, E. B. Hart, and C. A. Elvehjem, *J. Nutrition* **37**, 443 (1949).

[15] F. S. Daft, L. L. Ashburn, and W. H. Sebrell, *Science* **96**, 321 (1942).

[16] G. J. Martin, *Proc. Soc. Exptl. Biol. Med.* **51**, 353 (1952).

[17] E. Nielsen and C. A. Elvehjem, *J. Biol. Chem.* **145**, 713 (1942).

[18] F. W. Heumann, M. M. Krider, and H. G. Day, *Proc. Soc. Exptl. Biol. Med.* **52**, 257 (1943).

[19] A. D. Welch and L. D. Wright, *J. Nutrition* **25**, 555 (1943).

[20] G. A. Emerson and E. Wurtz, *Proc. Soc. Exptl. Biol. Med.* **57**, 47 (1944).

[21] L. D. Wright and A. D. Welch, *J. Nutrition* **27**, 55 (1944).

[22] K. R. Johanson, S. K. Shapiro, and W. B. Sarles, *J. Bacteriol.* **54**, 35 (1947).

[23] J. R. Couch, W. W. Cravens, C. A. Elvehjem, and J. G. Halpin, *J. Nutrition* **35**, 57 (1948).

[24] J. R. Couch, M. L. Sunde, W. W. Cravens, C. A. Elvehjem, and J. G. Halpin, *J. Nutrition* **37**, 251 (1949).

[25] F. W. Went and K. V. Thimann, Phytohormones, 294 pp. Macmillan Co., New York, 1937.

mainly on its biological effect in higher animals or microorganisms. No chemical and physical methods are available at the present time for the determination of biotin.

The microbiological methods have the advantage of being technically simple and short-term procedures. However, they have also serious limitations. Most of the microorganisms used for assay of biotin will respond only to free and not to bound biotin. Thus, concentrates must be prepared through enzymatic treatment or acid hydrolysis in order to free bound biotin and bring it into aqueous solution (see p. 529). Furthermore, microorganisms may also respond to close homologs, analogs, breakdown products, or precursors, such as pimelic acid, and to other seemingly unrelated chemicals, such as oleic acid (see p. 558) in a much more unspecific manner than higher animals do.

A. BIOASSAY USING HIGHER ANIMALS

PAUL GYÖRGY

The biological estimation of biotin may be carried out with rats and chicks made deficient in biotin by the use of special rations.

1. Rat Method

Under ordinary dietary conditions the biotin requirement of the growing or adult rat appears to be fully covered by the amount of biotin produced by the intestinal flora.[1] Incorporation of raw egg white (fresh or dried) or avidin in a synthetic experimental ration results in biotin deficiency by preventing the intestinal absorption of biotin which forms with avidin a stable unabsorbable complex (see p. 555). The gross manifestations of this egg white injury, synonymous with biotin deficiency, are characterized by cessation of growth and by a variety of symptoms involving fur and skin, such as "spectacled eyes," seborrheic dermatitis, alopecia, and other less specific or less regularly occurring manifestations.[2-4] Varying degrees of deficiency may be identified in the experimental animals. Rats showing cessation of growth, "spectacled eyes," or beginning alopecia are "incipiently deficient." Any combination of these symptoms coupled with more marked alopecia, closure of the eyes by exudate, and red, inflamed nose and snout characterizes a slightly to moderately deficient rat. Advanced loss of hair, spastic gait, beginning or advanced development of cracks and sores along with encrustation in the skin, scab formation, and seborrheic deposits classify the rat as moderately to severely deficient (Figs. 5 and 6).

[1] V. du Vigneaud and E. A. Evans, Jr., The Biological Action of the Vitamins, pp. 144ff. University of Chicago Press, Chicago 1942.

[2] M. A. Boas, *Biochem. J.* **21**, 712 (1927).

[3] P. György, *Z. ärztl. Fortbild.* **28**, 377, 417 (1931).

[4] P. György, *J. Biol. Chem.* **131**, 733 (1939).

A rat is considered severely deficient when it shows almost complete denudation of the skin, "kangaroo" gait, sores and cracking of the skin, particularly of the folds under the legs, closure of the eyes, and marked weight

FIG. 5. Biotin deficiency with scaly dermatosis, mainly in the neck and groins.

loss. There may be general exfoliation of the skin in small or larger scales (Figs. 7 and 8).

It is customary to base the bioassay of biotin in rats on the growth response, which is expressed more easily in quantitative terms. In contrast, the curative effect of biotin on cutaneous lesions and the other manifestations of biotin deficiency, although more specific than the growth effect, are difficult to assess exactly.

Weanling rats, preferably of the same sex and weighing not more than

35 to 40 g., are fed a suitable experimental ration.[5] At the end of a depletion period averaging 6 to 7 weeks, rats show cessation of growth and one or more of the specific symptoms of biotin deficiency. They are then assigned

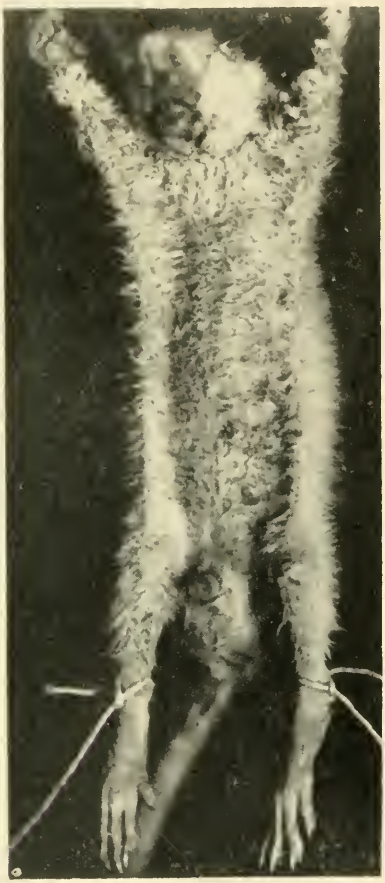


FIG. 6. Advanced scaly dermatosis in biotin deficiency involving the snout and whole venter.

to two or more dosage levels of the standard and of each unknown so that rats exhibiting the same degree of deficiency are distributed equally to each treatment. Pure synthetic biotin may serve as a reference standard in two or more dilutions. To insure their availability to the rat, despite the avidin in the diet, the biotin supplements may be given by injection rather

[5] D. M. Hegsted, J. J. Oleson, R. C. Mills, C. A. Elvehjem, and E. B. Hart, *J. Nutrition* **20**, 599 (1940).

FIG. 7. Almost generalized scaly dermatosis with severe alopecia.

FIG. 8. Exfoliative dermatitis in very severe biotin deficiency.

than by mouth. A test period of 4 weeks is customary, and the results are computed as in other growth assays. Gain in weight per week during the test period is a linear function of the log-dose of biotin within the range of 0.1 to 1.0 γ per day.[6]

Male rats are more suceptible to biotin deficiency than female rats,[2-4] and albino rats more than piebald or black rats.[2, 4, 7]

2. Chick Method

The chick requires much larger amounts of biotin than the rat, and even under normal conditions the intestinal synthesis of biotin in chicks is too limited so that biotin deficiency can be produced by a biotin-low diet alone, without the aid of avidin.[5, 8] Biotin deficiency in chicks mainfests itself as in rats by a declining weight curve and by cutaneous lesions, especially on the bottoms of the feet and around the beak.

The technique used for the biological assay of biotin in rats may also be applied to chicks, with the difference that a biotin-free purified ration is used.[6] Successful assays may also be obtained with a commercial chick ration mixed with egg white (avidin to inactivate its biotin content). The assay may begin without a prolonged depletion period and is usually more of prophylactic nature, lasting 4 weeks. Relative potency is computed from growth response and is supported by evidence of dermatis in the negative controls and its prevention at all dosage levels of biotin, except possibly the lowest.

B. MICROBIOLOGICAL ESTIMATION

ESMOND E. SNELL

A wide variety of microorganisms require biotin for growth, and many of these have been used for its assay. These include *Clostridium butylicum*, several lactic acid bacteria and yeasts, *Rhizobium trifolii, and Neurospora crassa*. Each of the various proposed methods has been summarized elsewhere in some detail.[9] Of these, the most widely used and uniformly successful has been the method of Wright and Skeggs,[10] which employs *Lactobacillus arabinosus* as the test organism. [9, 11, 12] Growth of *L. arabinosus*

[6] P. György, Vitamin Methods, Vol. 2, pp. 228ff. Academic Press, New York, 1951.
[7] H. T. Parsons and E. Kelly, *J. Biol. Chem.* **100**, 645 (1933).
[8] S. Ansbacher and M. Landy, *Proc. Soc. Exptl. Biol. Med.* **48**, 3 (1941).
[9] E. E. Snell, *in* Vitamin Methods, Vol. 1, p. 327. Academic Press, New York, 1950.
[10] L. D. Wright and H. R. Skeggs, *Proc. Soc. Exptl. Biol. Med.* **56**, 95 (1944).
[11] L. D. Wright, *in* Biological Symposia, XII, Estimation of the Vitamins, p. 290. Jaques Cattell Press, Lancaster, Pa., 1947.
[12] Association of Vitamin Chemists, Methods of Vitamin Assay, 2nd ed., p. 245. Interscience Publishers, New York, 1951.

in the biotin-free basal medium used increases with the biotin concentration in the range from 0 to about 2 mγ per 10 ml. of medium. Pure biotin and samples to supply biotin at several levels within this range are added to individual tubes containing 5 ml. of the double-strength basal medium; each tube is then diluted to 10 ml., capped, autoclaved, cooled, and inoculated. Response of the test organism is customarily determined by acid titration after 72 hours of incubation at 30 to 37°, or turbidimetric estimations of growth can be made as early as 24 hours.

The specificity of the test organism to various derivatives of biotin which might occur naturally has been summarized elsewhere.[11] None of these, including soluble bound forms of biotin, such as biocytin,[13] or the more complex insoluble combinations of this vitamin that occur naturally, can support growth in place of biotin. It is necessary, therefore, to liberate biotin from these combinations by hydrolysis if figures from the total biotin content of natural materials are desired. In most products, hydrolysis with 6 N H_2SO_4 at 120° for 1 hour liberates a maximum amount of biotin; in some products, however, this treatment destroys some biotin.[9, 11] Some investigation of extraction procedures should therefore be made prior to extensive study of any given product. Hydrochloric acid destroys biotin under some conditions and should not be used in place of sulfuric acid. Unsaturated fatty acids, such as oleic and linoleic acids, replace biotin for *L. arabinosus* when present in high amounts[14] and interfere with biotin assay in considerably lower concentration. They are readily removed by filtration of the acid-hydrolyzed sample through paper, or by ether extraction.[9]

Yeast assay methods for biotin are somewhat simpler and require less time than that using *L. arabinosus*, but require more complex apparatus and turbidimetric estimation of growth. An unpublished procedure of Atkin and coworkers[9] gives excellent results, as does that of Hertz.[9, 15] The same extraction procedures used for *L. arabinosus* are required for liberation of biotin. Yeasts are less specific than lactic acid bacteria in their response to biotin; biotin sulfoxide, for example, which may occur naturally in rancid foods, is active in place of biotin.[9] Fatty acids, on the other hand, do not replace biotin for yeast and thus show less interfering action when this procedure is used.

By slight modifications in technique, including aseptic addition of the unheated samples, any of the methods recommended above can be adapted to determination of avidin, the biotin-binding protein of egg white. In

[13] L. D. Wright, E. L. Cresson, H. R. Skeggs, R. L. Peck, D. E. Wolf, T. R. Wood, J. Valiant, and K. Folkers, *Science* **114**, 635 (1951).

[14] H. P. Broquist and E. E. Snell, *J. Biol. Chem.* **188**, 431 (1951).

[15] R. Hertz, *Proc. Soc. Exptl. Biol. Med.* **52**, 15 (1943).

such procedures, a constant, known amount of biotin is added to each of several tubes, and the amount of protein required to render a given proportion of it unavailable to the test organism is determined.[16]

VIII. Standardization of Activity

PAUL GYÖRGY

Biotin is available in pure synthetic form. This *dl*-biotin represents the best standard for reference purposes, if the fact is considered that for animals the activity of the synthetic product is only 50% that of the natural *d*-biotin.

In microbiological tests the biotin effect is less specific and may be exerted by a large number of chemically closely related, but also chemically different, compounds such as oleic acid and pimelic acid. All these effects may be expressed in biotin equivalents.

IX. Occurrence in Food

PAUL GYÖRGY

Biotin is widely represented in various food products. Its estimation in microbiological tests has great limitations, owing mainly to two interfering factors: (a) the occurrence of chemical compounds with unspecific biotin activity; (b) the difficulty of complete extraction of the bound biotin fraction (see p. 529).

In animal assay these complications are more or less eliminated, biotin activity being exerted mainly by its true chemical equivalent and by the liberation of biotin in the digestive tract followed by its absorption and utilization.

Using rats fed an egg white diet, György[1] found liver, kidney, yeast, and, to a much lesser extent, cow's milk and bananas to be good sources of biotin. In contrast, brain, spleen, thymus, blood, heart, peripheral muscle (beef), and rice polish have shown, at least in the doses used, no biological biotin activity. For instance, beef liver (fresh) was therapeutically active in daily doses of 0.5 g., kidney in 1.0 g., yeast (dry) in 0.3 g., cow's milk in 10 ml., and bananas in 5 g. Heart was given in daily doses of up to 3.0 g., muscle

[16] R. E. Eakin, E. E. Snell, and R. J. Williams, *J. Biol. Chem.* **140**, 535 (1941).

[1] P. György, *J. Biol. Chem.* **131**, 733 (1939).

up to 7.5 g., brain, spleen, and thymus up to 2.0 g., and rice polish up to 0.6 g. Cow's milk was not so potent in winter as in summer. Breast milk had only very slight therapeutic potency in egg white injury; as much as 25 ml. daily had no constant effect.

Most analytical data on the distribution of biotin in food products were obtained by microbiological tests. In general, the agreement between the findings of various workers is only fair, owing probably to differences in the methods of extraction and to the particular test organisms used. The liberation of free biotin from its bound form was attempted by autoclaving with water or by acid hydrolysis[2] or by enzymatic treatment.[3-5]

The microbiological tests confirmed in general the findings obtained in animal assays.[6] Biotin concentrations were found to be appreciably lower in human milk than in cow's milk. The biotin content of milk from animals of different species is given in Table X.

TABLE X

Biotin Content of Milk from Mammals of Different Species, γ/ml.

	Human (white)	Mare (thoroughbred)	Cow (Jersey, Guernsey)	Goat (Sanaan, Nubian, Toggenberg)	Dog (English bull)	Mouse (albino dba, C_3H)
Biotin content	0.001	0.022	0.050	0.063	0.12	0.32
Number of samples	8	1	30	4	1	3
Number of specimens	4	1	15	4	1	3
Maximum and minimum values	0.005–<0.001		0.11–0.016	0.083–0.047		0.48–0.22

In other series[7] the biotin content of cow's milk, determined microbiologically, was found to vary between 0.011 to 0.037 γ per milliliter, the value rising to a maximum after the first few days and then falling.

In later European studies[8] the biotin content of human milk was found to range from a trace to 0.033 γ per milliliter with an average value of 0.0016, in good accord with the figures given in Table X. Intramuscular injection of 5 mg. caused a marked rise in the biotin content of breast milk, up to 0.768 γ per milliliter in one subject. The level fell after a few hours, and the effect of the large dose disappeared within 3 days.

[2] J. O. Lampen, G. P. Bahler, and W. H. Peterson, *J. Nutrition* **23**, 11 (1942).

[3] V. H. Cheldelin, M. A. Eppwright, E. E. Snell, and B. M. Guirard, *Univ. Texas Publ.* **4237**, 15 (1942).

[4] R. J. Williams, V. H. Cheldelin, and H. K. Mitchell, *Univ. Texas Publ.* **4237**, 97 (1942).

[5] V. H. Cheldelin and R. J. Williams, *Univ. Texas Publ.* **4237**, 105 (1942).

[6] P. György, D. B. Melville, D. Burk, and V. du Vigneaud, *Science* **91**, 243 (1940).

[7] J. M. Lawrence, B. L. Herrington, and L. A. Maynard, *J. Nutrition* **32**, 73 (1946).

[8] W. Neuweiler and W. Ritter, *Intern. Z. Vitaminforsch.* **21**, 239 (1949).

TABLE XI

BIOTIN CONTENT OF FOODS, γ/G.

Product	Fresh	Dry
Cereal		
Whole wheat	0.052	0.058
Bread, whole wheat	0.019	0.029
Bread, white, unenriched	0.011	0.016
Flour, white, unenriched	0.007	0.008
Corn meal, white	0.058	0.066
Hominy grits	0.006	0.007
Rice Crispies	0.013	0.014
Dairy		
Milk, whole	0.050	0.41
Cheese	0.036	0.054
Eggs	0.090	0.35
Beef		
Round	0.026	0.077
Liver	0.96	2.6
Heart	0.081	0.34
Brain	0.074	0.33
Veal, chop	0.020	0.064
Pork		
Loin (lean) I	0.038	0.13
Loin (lean) II	0.020	0.074
Bacon	0.074	0.095
Ham (lean)	0.040	0.12
Lamb, leg	0.021	0.069
Mutton, shoulder	0.027	0.064
Chicken		
Leg	0.098	0.44
Breast	0.054	0.21
Sea foods		
Halibut	0.080	0.30
Salmon	0.053	0.19
Oyster	0.087	0.44
Vegetables		
Beans, dried lima	0.098	0.11
Beets	0.003	0.022
Beet greens	0.027	0.26
Cabbage	0.024	0.29

TABLE XI—*Continued*

Product	Fresh	Dry
Vegetables—*Continued*		
Carrots	0.025	0.21
Cauliflower	0.17	1.5
Lettuce	0.031	0.60
Mushrooms	0.16	1.3
Okra	0.055	0.54
Onions, dry	0.035	0.28
Peas, dried English	0.18	0.20
Peas, black-eyed	0.21	0.23
Peas, green English	0.094	0.38
Potatoes, Irish	0.006	0.027
Potatoes, sweet	0.043	0.14
Spinach	0.069	0.67
Turnips	0.021	0.26
Fruits		
Apples	0.009	0.062
Bananas	0.044	0.18
Cantaloupe	0.031	0.32
Grapefruit	0.030	0.27
Oranges	0.019	0.15
Peaches (frozen)	0.017	0.062
Raisins	0.031	0.041
Strawberries	0.040	0.40
Tomatoes	0.040	0.67
Watermelon	0.036	0.36
Miscellaneous		
Chocolate	0 32	0.32
Molasses	0.091	0.12
Roasted peanuts	0.34	0.35
Sugar	<0.004	—
Royal jelly	1.7	5.4

Microbiological tests with various food products confirmed the high biotin content of liver and kidney, and its low concentration in meat (beef and pork). On a dry weight basis, egg, most fresh vegetables, and several fruits are also good sources of biotin whereas wheat and corn products are consistently poor.[2, 5] Biotin occurs in a large variety of seeds.[9] Oat seedlings contain somewhat larger amounts in the root and coleoptile tips than in the roots. Although in general not a good source of biotin, meat contains some (cow, calf, pork, hen, etc.) and a high proportion remains after cooking.[10]

[9] F. Kögl and A. J. Haagen-Smit, *Hoppe-Seyler's Z. physiol. Chem.* **243**, 209 (1936).
[10] B. S. Schweigert, E. Nielsen, J. M. McIntire, and C. A. Elvehjem, *J. Nutrition* **26**, 65 (1943).

The biotin content of fresh cheese, with a range of 0.011 to 0.076 γ per gram increases two- or threefold on ripening, through bacterial synthesis.[11]

It is claimed that royal jelly is very rich in biotin, containing 4.1 γ per gram; pollen and honey contain 0.25 and 0.00066 γ per gram, respectively.[12]

The biotin content of various food products as determined by Cheldelin and Williams[5] in a large selection of samples for each food is given with average values in Table XI.

Avidin, the only natural antibiotin (see p. 555) occurs in raw egg white and therefore would interfere with the estimation of biotin in whole raw egg. Quantitative determinations of biotin and avidin in whole egg in which egg white and egg yolk are thoroughly mixed invariably show an excess of avidin, as illustrated by the following example:[13]

Avidin total, free (A) + bound (AB) γ/ml.	Avidin in form of biotin complex (AB) γ/ml.	Excess of free avidin (A) γ/ml.
0.50	0.19	0.31

This conclusion was confirmed[14] indirectly by experiments in which egg white, egg yolk (of one egg), and mixtures of yolk and white in the proportion found in the whole egg were analyzed, as shown by the following values:

		Biotin (free *plus* bound)			Avidin (free *plus* bound)		
		Found		Calculated	Found		Calculated
	Vol., ml.	γ/ml.	γ/total vol.	γ/total vol.	γ/ml.	γ/total vol.	γ/total vol.
White	27	0.15	4.1		0.70	19.0	—
Yolk	14	0.53	7.4		0.0	0.0	—
Whole egg	41	0.30	12.3	11.5	—	22.0	19.0

The biotin of egg yolk is present in a high molecular undialyzable and in the same time microbiologically active form (p. 555). This may explain its independent existence in the face of excess of avidin in the surrounding egg white.

[11] R. A. Sullivan, E. Nielsen, and J. Jarmol, *J. Nutrition* **25**, 463 (1943).
[12] G. Kitzes, H. A. Schuette, and C. A. Elvehjem, *J. Nutrition* **26**, 241 (1943).
[13] P. György and C. S. Rose, *Proc. Soc. Exptl. Biol. Med.* **49**, 294 (1942).
[14] V. P. Sydenstricker, *Semana méd. español.* **6**, 356 (1943).

X. Effects of Deficiency

PAUL GYÖRGY

A. RATS AND MICE

Egg white injury as a clinical manifestation of biotin deficiency was first produced in rats, and until the present time rats have been the preferred experimental animals for the study of this deficiency. As stated in previous sections biotin deficiency produced by feeding raw or unheated commercial egg white to rats is characterized by a progressive dermatitis (see Figs. 5–7), reminiscent of scaly desquamative dermatoses of the seborrheic type.[1-3]

The initial symptom of a progressive disturbance of the skin in rats is a dermatitis which is at first confined to the groin and the genitalia and to the neck and around the snout. In the later stage of the disease in rats, brown, adherent scales of varying size and thickness, together with progressive alopecia, become apparent in and around the inflamed areas. As a rule, neither the dermatitis nor the alopecia in these rats has well-defined borders. Often small, thin, almost point-like but also confluent, brown pigmented scales appear, usually confined to the back and not necessarily accompanied by severe dermatitis or alopecia, although both may be present simultaneously with erythematous lesions.[1-5] The region round the mouth is usually very severely affected. In some instances skin hemorrhages and edema, especially of the feet, appear.[4, 6] In mild cases the skin lesions may be limited to alopecia around the eyes: "spectacle eye condition."[7]

In exceptional cases the disease may involve the skin of the whole body of the rat and may resemble the typical exfoliative dermatitis seen in adults or Leiner's disease (erythroderma desquamativum), its counterpart in infants. The epidermis of the rat is shed in large or small thin scales, (see Fig. 8), which on account of their rapid and continuous production become colorless and devoid of fat, the brown color and the high fat content being confined to the first, long adherent scales. Because of blepharitis, the eyelids often stick together and are covered with yellow scales. Generalized alopecia and pruritus are often observed. In black or piebald rats depigmentation of the fur becomes noticeable, and the black hair turns brownish

[1] P. György, *Z. ärztl. Fortbild.* **28**, 377, 417 (1931).
[2] P. György, *in* Handbuch der Kinderheilkunde, 4th ed., Chapter 10, p. 45. F. C. W. Vogel, Berlin, 1935.
[3] P. György, *Arch. Dermatol. and Syphilol.* **43**, 230 (1941).
[4] M. A. Boas, *Biochem. J.* **21**, 712 (1927).
[5] H. T. Parsons, *J. Biol. Chem.* **90**, 351 (1931).
[6] W. D. Salmon and J. G. Goodman, *J. Nutrition* **8**, 1 (1934).
[7] E. Nielsen and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.* **48**, 349 (1941).

or even gray.[2, 3, 8, 9] Excoriations heal slowly. Cutaneous abscesses are rare, and when present they are ecthyma-like. Mild, sublingual ulcers are fairly common. A spastic gait and a kangaroo-like posture,[1] due probably to hypertonicity of the striated muscles[2, 3] particularly of the hind legs, are characteristic symptoms of egg white injury in the advanced stage. Biotin deficiency in rats may cause retention of the testes in the abdomen and even a return of the already descended organs to the abdomen, owing to the contracture of the cremaster muscle.[10]

Biotin is needed for successful gestation and is probably a necessary factor in lactation both in rats[11] and in mice.[12]

The skin lesions of biotin deficiency become more severe on low-fat rations than on diets containing 18% of butterfat or hydrogenated cottonseed oil or 0.2 ml. of linseed oil.[6]

Biotin deficiency of a minor degree (combined with manifestations of folic acid deficiency) may also be induced in the rat by feeding of sulfonamide-containing purified rations (p. 588).[13-18] No acceleration or aggravation of biotin deficiency was observed when succinylsulfathiazole was added to a diet containing egg white.[18]

Biotin deficiency in black mice is characterized by depigmentation of the fur and alopecia but not so much by scaly dermatitis.[19-21]

The manifestations of biotin deficiency in rats and mice may be cured in 2 to 4 weeks by the administration of biotin or food products containing biotin. Severe skin lesions require a prolonged period of treatment. Depigmentation of the fur may not completely respond to biotin.[9]

There appears to be a connection between biotin deficiency and pantothenic acid deficiency, and the depigmentation of fur first recognized in deficiency of pantothenic acid[19, 22, 22a, 23] may be the outward sign of such an

[8] M. Sullivan and J. Nicholls, *Arch. Dermatol. and Syphilol.* **45**, 295 (1942).
[9] G. A. Emerson and J. C. Keresztesy, *Proc. Soc. Exptl. Biol. Med.* **51**, 358 (1942).
[10] W. K. Manning, *Science* **112**, 89 (1950).
[11] C. Kennedy and L. S. Palmer, *Arch. Biochem.* **7**, 9 (1945).
[12] L. Mirone and L. R. Cerecedo, *Arch. Biochem.* **15**, 324 (1947).
[13] F. S. Daft, L. L. Ashburn, and W. H. Sebrell, *Science* **96**, 321 (1942).
[14] G. J. Martin, *Proc. Exptl. Biol. Med.* **51**, 353 (1942).
[15] E. Nielsen and C. A. Elvehjem, *J. Biol. Chem.* **145**, 713 (1942).
[16] F. W. Heumann, M. M. Krider, and H. G. Day, *Proc. Soc. Exptl. Biol. Med.* **52**, 257 (1943).
[17] A. D. Welch and L. D. Wright, *J. Nutrition* **25**, 555 (1943).
[18] G. A. Emerson and E. Wurtz, *Proc. Soc. Exptl. Biol. Med.* **57**, 47 (1944).
[19] P. György and C. E. Poling, *Proc. Soc. Exptl. Biol. Med.* **45**, 773 (1940).
[20] E. Nielsen and A. Black, *J. Nutrition* **28**, 203 (1944).
[21] J. W. Wilson, E. H. Leduc, and D. H. Winston, *J. Nutrition* **38**, 73 (1949).
[22] P. György and C. E. Poling, *Science* **92**, 202 (1940).
[22a] P. György, C. E. Poling, and Y. SubbaRow, *J. Biol. Chem.* **132**, 789 (1940).
[23] K. Unna, G. V. Richards, and W. L. Sampson, *J. Nutrition* **22**, 553 (1941).

interrelationship. The symptoms of biotin deficiency induced in young rats by administration of succinylsulfathiazole may be aggravated by simultaneous pantothenic acid deficiency.[18] Addition of biotin to the diet not only will protect the animals against specific changes of biotin deficiency but will also reduce the severity of the symptoms of pantothenic acid deficiency. In rats receiving a highly purified diet including thiamine, riboflavin, and pantothenic acid and supplemented with succinylsulfathiazole, severe signs of pantothenic acid deficiency developed.[24] The symptoms were accompanied by a marked reduction in the pantothenic acid content of the liver and were relieved by administration of biotin and folic acid. Thus, the utilization of pantothenic acid seems to depend on the presence of biotin and folic acid, apparently synthesized in the gut by bacteria.

B. CHICKS AND TURKEYS

In chicks and turkeys, dermatitis and perosis are the chief manifestations of biotin deficiency induced by feeding egg white or simply by an experimental ration low in biotin.[25-31] The lesions usually start to appear at 2 to 3 weeks and become quite severe at 4 weeks. The syndrome is characterized by lesions, first appearing on the bottom of the feet, followed by mandibular lesions. On a biotin-free synthetic diet not containing egg white, dermatitis may be regularly produced, but the onset and appearance of perosis is often erratic. The addition of raw egg white to the ration will enhance the development of perosis.[32]

Hatchability of eggs is considerably reduced in biotin deficiency and may be restored by feeding the hen a ration rich in biotin.[33-35]

Prevention of perosis requires less biotin than prevention of dermatitis

[24] L. D. Wright and A. D. Welch, *Science* **97**, 426 (1943).
[25] W. H. Ott, *J. Biol. Chem.* **157**, 131 (1945).
[26] L. W. McElroy and T. H. Jukes, *Proc. Soc. Exptl. Biol. Med.* **45**, 296 (1940).
[27] S. Ansbacher and M. Landy, *Proc. Soc. Exptl. Biol. Med.* **48**, 3 (1941).
[28] D. M. Hegsted, R. C. Mills, G. M. Briggs, C. A. Elvehjem, and E. B. Hart, *J. Nutrition* **23**, 175 (1942).
[29] T. H. Jukes and F. H. Bird, *Proc. Soc. Exptl. Biol. Med.* **49**, 234 (1942).
[30] H. Patrick, R. V. Boucher, R. A. Dutcher, and H. C. Knandel, *Proc. Soc. Exptl. Med.* **48**, 456 (1941).
[31] H. Patrick, R. V. Boucher, R. A. Dutcher, and H. C. Knandel, *J. Nutrition* **26**, 197 (1943).
[32] L. R. Richardson, A. G. Hogan, and O. N. Miller, *Univ. Missouri Agr. Expt. Stat. Research Bull.* p. 343 (1942).
[33] W. W. Cravens, E. E. Sebesta, J. G. Halpin and E. B. Hart, *Proc. Soc. Exptl. Biol. Med.* **50**, 101 (1942).
[34] W. W. Cravens, W. H. McGibbon, and E. E. Sebesta, *Anat. Record* **90**, 55 (1944).
[35] J. R. Couch, W. W. Cravens, C. A. Elvehjem, and J. G. Halpin, *Arch. Biochem.* **21**, 77 (1949).

or support of normal growth.[29] This may be achieved by the addition of 2 to 5 γ of biotin per day or by feeding 7 to 10 γ of biotin per 100 g. of diet.[28]

C. DOGS

Puppies fed a synthetic diet deficient in the vitamin B complex but supplemented with all known members of this complex with the exception of biotin and folic acid developed a progressive paralysis after 7 to 48 weeks. This neurological condition responded, at least temporarily, to injections of biotin.[36, 37] Later it was shown that the symptoms were due primarily to a potassium deficiency, and a single adequate dose of potassium was followed by complete remission lasting for 6 to 10 weeks. Here an apparently latent potassium deficiency was accentuated by simultaneous deficiency of biotin.[38, 39]

D. PIGS

Egg white injury may also be produced in pigs by feeding the usual experimental diet containing about 30% commercial dry egg.[40] Biotin deficiency in a mild form may develop[41] in young piglets on a biotin-free ration without the addition of avidin (egg white). The syndrome in pigs is characterized by alopecia, seborrheic skin changes, spasticity of the hind legs, and cracks in the feet. The same condition, in less pronounced form perhaps, may also be produced by administration of phthalylsulfathiazole (and not of sulfaguanidine) in a semisynthetic diet.[42] In both instances biotin proved to be the specific factor of prevention or treatment.[40-42]

E. COWS

It has been claimed[43] that the dairy calf requires an exogenous supply of biotin. In the absence of biotin, paralysis of the hind legs was observed. The paralysis was cured by the administration of biotin.

F. FISH

In young trout, biotin deficiency manifests itself by progressive anemia[44] and the so-called "blue slime" disease.[45] In young brown trouts fed a ra-

[36] S. G. Smith, *Science* **100**, 389 (1944).
[37] S. G. Smith and T. E. Lester, *Am. J. Physiol.* **144**, 175 (1945).
[38] W. R. Ruegamer, C. A. Elvehjem, and E. B. Hart, *Proc. Soc. Exptl. Biol. Med.* **61**, 234 (1946).
[39] S. G. Smith, *Proc. Soc. Exptl. Biol. Med.* **63**, 339 (1946).
[40] T. J. Cunha, D. C. Lindley, and M. E. Ensminger, *J. Animal Sci.* **5**, 219 (1946).
[41] W. P. Lehrer, Jr., A. C. Wiese, and P. R. Moore, *J. Nutrition* **47**, 203 (1952).
[42] D. C. Lindley and T. J. Cunha, *J. Nutrition* **32**, 47 (1946).
[43] A. C. Wiese, B. C. Johnson, and W. B. Nevens, *Proc. Soc. Exptl. Biol. Med.* **62**, 521 (1946).
[44] B. A. McLaren, E. B. Keller, D. J. O'Donnell, and C. A. Elvehjem, *Arch. Biochem.* **15**, 169 (1947).

tion containing egg white, the disease will develop in about 12 weeks. Biotin and biotin-containing food will correct the deficiency.

G. MONKEYS

Chronic biotin deficiency in monkeys produces a thinning of the fur with accompanying progressive depigmentation of the hair.[46] Chronic and more acute biotin deficiency may be cured in monkeys by biotin or biotin-containing food products.[46, 47]

H. MAN

The production of biotin deficiency has been attempted in human volunteers.[48, 49] Four volunteers were fed a diet containing minimal amounts of biotin and 30 % of the total caloric intake in the form of egg white. Temporary fine scaly desquamations of the skin without pruritus appeared during the third and fourth week; more marked maculosquamous dermatitis became evident around the seventh week on the neck, hands, arms, and legs of one of the volunteers. Later, during the ninth and tenth week, all patients showed fine branny desquamation, accompanied by mild depression followed by extreme lassitude, somnolence, muscle pains, and hyperesthesia. After the tenth week anorexia with occasional nausea became evident. Slight anemia, a large increase of the serum cholesterol level, and a smaller rise in bile pigments were noticed. The urinary excretion of biotin was reduced after 7 to 8 weeks to 3.5 to 7.3 γ per day, as compared with 29 to 52 γ per day on a normal diet. Injection of biotin in doses of 75 to 300 γ per day was followed by prompt relief of the symptoms in 3 to 5 days, and the urinary excretion of biotin rose in the same time to 55 γ per day.

Although the clinical syndrome observed in these volunteers receiving a diet low in protein was characterized by mild and rather unspecific clinical manifestations, the observations recorded seem to favor the assumption that biotin is an essential nutrient for man. The figures of urinary excretion are in accord with this view.

Less conclusive, although perhaps more interesting, is the observation of an exfoliative dermatitis of several years duration in a 66-year-old patient with poor dietary habits.[50]

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[45] A. M. Phillips, Jr., and E. O. Rodgers, *Progressive Fish Culturist* **12**, 67 (1950).

[46] H. A. Waisman, K. B. McCall, and C. A. Elvehjem, *J. Nutrition* **29**, 1 (1945).

[47] J. G. Lease, H. T. Parsons, and E. Kelly, *Biochem. J.* **31**, 433 (1937).

[48] V. P. Sydenstricker, S. A. Singal, A. P. Briggs, N. M. de Vaughn, and H. Isbell, *Science* **95**, 176 (1942).

[49] V. P. Sydenstricker, S. A. Singal, A. P. Briggs, N. M. de Vaughn, and H. Isbell, *J. Am. Med. Assoc.* **118**, 1199 (1942).

[50] R. H. Williams, *New Engl. J. Med.* **228**, 247 (1943).

"Since adolescence the patient had been extremely fond of raw eggs, putting one or two into each glass of wine that he took. For several decades he had drunk wine or whiskey almost every day. During the six years preceding admission, he had drunk from 1 to 4 quarts of wine daily. In order to have a sufficient number of eggs for his drinks, he deserted his family and moved to the country so that he could maintain his own chicken farm. During this period of time he ate from two to six dozen raw eggs per week. He did not eat at any regular time. Sometimes he ate only one or two meals a day, and sometimes he drank nothing but wine and eggs for 1 or 2 days. His choice of foods was narrow and consisted chiefly of canned goods; rarely did he drink milk or eat liver (sources of biotin).

"So long as he could remember, his skin had been quite red, but 5 years previously the redness increased and many scales were noted. A mild conjunctivitis had been present for several months. These changes persisted, and 3 months before admission there was an increase in their severity. There was not much fluctuation in the nature of the rash. There was never any itching, vesicles, bullas or evidence of skin infection; nor was there diarrhea, sore tongue, neuralgia, cheilosis, night blindness or hemorrhagic phenomena."

The patient with his marked exfoliative dermatitis has undergone a partial, and later a more radical, amputation of the penis because of a carcinoma. This was followed by prolonged infection of the urinary tract and pneumonia, treated in the hospital. During his hospitalization the patient received a well-balanced, rich diet with supplements of vitamins. The exfoliative dermatitis, with all its manifestations, improved rapidly, and the skin regained its outwardly normal appearance. An attempt to reactivate the dermatitis by giving the patient a diet low in biotin failed.

Unquestionably this patient must have consumed for several years a diet conducive to the production of biotin deficiency. In raw whole egg, which, in addition to wine, comprised the bulk of the patient's daily diet, avidin, the natural antibiotin, is present in excess of biotin.[51] The exfoliative dermatitis which the patient exhibited for several years is consistent with an underlying deficiency of biotin. However, in the absence of a specific therapeutic response and in view of the failure to reactivate the disease after it subsided, the diagnosis of biotin deficiency in this case cannot be maintained with certainty.

The claim that egg white injury in rats as manifestation of biotin deficiency is the analog of acrodynia ("pink disease") seen in infants and in young children[52] is not supported by clinical comparison or by any other available criterion, including therapeutic tests.

The similarity of the cutaneous manifestations seen in experimental biotin deficiency in animals, especially in rats, with the syndrome of seborrheic dermatitis and—in its most pronounced form—with Leiner's disease (erythroderma desquamativum or exfoliative dermatitis) in young infants has

[51] P. György and C. S. Rose, *Proc. Soc. Exptl. Biol. Med.* **49**, 294 (1942).

[52] L. M. Findlay and R. O. Stern, *Arch. Disease Childhood* **4**, 1 (1929).

been pointed out by several observers.[1-3, 53-56] Leiner's disease is seen often in breast-fed infants, frequently in association with persistent diarrhea. The relatively low biotin content of human milk (p. 596), together with possible secondary loss of biotin due to the diarrhea, and perhaps also the changed intestinal flora, have been linked with possible biotin deficiency in this pathological condition. It has been also stated[54, 56] that the urinary excretion of biotin is reduced in infants with seborrheic dermatitis compared with normal infants or with infants showing eczematous cutaneous lesions. The saturation test with biotin seemed to indicate a state of biotin depletion in infants with seborrheic dermatitis.[56] Finally, it has been claimed that seborrheic dermatitis and even Leiner's disease is beneficially influenced by biotin.[54-56] However, it should be pointed out that neither the above metabolic findings nor these therapeutic results have received confirmation on a larger scale. As a matter of fact, in the observation of the author (unpublished) biotin appeared to be without any uniformly regular beneficial effect in seborrheic dermatitis or in Leiner's disease. The positive results recorded[54-56] might have been purely coincidental. Thus, the relation of biotin to seborrheic dermatitis in infants and children, and in general the indications for its use in any cutaneous disease in man, remain obscure.

I. FAT INFILTRATION OF THE LIVER

It has been claimed that biotin produces a fatty liver characterized by a relatively high content of cholesterol[57, 58] and that "choline has no appreciable effect in preventing biotin fatty livers."[59, 60] In contrast, both inositol and lipocaic counteracted this type of fat depositions.[59] Later this statement was modified, and at least partial response to choline was admitted.[61] In these experiments "the biotin fatty liver" was induced in rats after a preliminary period of vitamin depletion followed by administration of biotin. Best and his associates[62] have called attention to the fact that vitamin depletion during the preliminary period was apparently less important than a lowered caloric intake which accompanies withdrawal

[53] A. Brown, *Glasgow Med. J.* **29**, 309 (1948).
[54] J. Svejcar and J. Homolka, *Ann. Paediat.* **174**, 175 (1950).
[55] V. Tripputi, *Lattante* **21**, 78 (1950); cf. *Nutrition Abstr. & Revs.* **20**, 459 (1950).
[56] H. Berger, *Intern. Z. Vitaminforsch.* **22**, 190 (1950).
[57] E. W. McHenry and G. Gavin, *J. Biol. Chem.* **140**, LXXXVII (1941).
[58] G. Gavin and E. W. McHenry, *J. Biol. Chem.* **141**, 619 (1941).
[59] G. Gavin, J. M. Patterson, and E. W. McHenry, *J. Biol. Chem.* **148**, 275 (1943).
[60] E. W. McHenry and J. M. Patterson, *Physiol. Revs.* **24**, 128 (1944).
[61] M. L. MacFarland and E. W. McHenry, *J. Biol. Chem.* **159**, 605 (1945).
[62] C. H. Best, C. C. Lucas, J. M. Patterson, and J. H. Ridout, *Biochem. J* **40**, 368 (1946).

of vitamins from the diet. Further, the same authors have also shown (a) that biotin will not regularly produce fatty liver, (b) that if fatty liver develops there is no selective deposition of cholesterol esters in the liver, (c) that inositol has no specific effect on bound cholesterol, and (d) that the fatty liver observed when biotin is administered is by no means resistant to choline The effect of inositol is synergistic to that of choline, the latter being the "primary" and more effective lipotropic agent. Even after administration of biotin the usual constant relationship between the accumulation of cholesterol esters and the deposition of neutral fat in the liver remains unchanged. Best and his associates[62] have found no evidence of any specific effect of choline, inositol, and biotin on the absolute amount of free cholesterol or phospholipids in the liver, and they recommended in the light of all their pertinent findings that the term "biotin fatty liver" be abandoned.

The role of biotin in fat metabolism and indirectly the problem of "biotin fatty liver" has been recently revived by Okey and her associates.[63 65] Rats fed a diet moderately rich in egg white or dried whole egg (avidin) will develop a mild degree of biotin deficiency, characterized among other specific manifestations of the deficiency condition by a reduced content of fat and in particular by that of cholesterol in the liver. In severe stages of the deficiency the fat depots of the subcutaneous tissue and of all the viscera will rapidly disappear. Addition of biotin to the diet will lead to reaccumulation of fat in the fat depots and in the liver. In cases of mild biotin deficiency the reappearance of fat in the liver after administration of biotin may be observed without any significant change in food intake. In rats fed experimental rations enriched in cholesterol (through dried whole egg or cholesterol added to egg white) and supplemented with ample biotin, the liver fatty acid values were one and one-half to three times, and total liver cholesterol values six to eight times, as high as those of rats fed similar adequate cholesterol-free diets. Most of the increase in cholesterol was in the esterified fraction. The same rations, low in biotin, rich in cholesterol, and without extra supplements of biotin, will not support storage of fat or cholesterol in the liver.

Avidin given with the diet will effectively prevent the deposition of excess cholesterol in the liver. In contrast, no definite indication was obtained[65] for a "therapeutic" effect of avidin, i.e., for the disappearance of cholesterol stores in the liver, which were accumulated prior to the administration of avidin.

In view of the fact that in biotin deficiency cholesterol esters show a

[63] R. Okey, *J. Biol. Chem.* **165**, 383 (1946).

[64] R. Okey, R. Pencharz, and S. Lepkovsky, *Am. J. Physiol.* **161**, 1 (1950).

[65] R. Okey, R. Pencharz, S. Lepkovsky, and E. R. Vernon, *J. Nutrition* **44**, 83 (1951).

preferential accelerated rate of reduction over that of total cholesterol, it may be assumed that[65] biotin plays a role mainly in the esterification and perhaps even in the formation and storage of fatty acids, and only incidentally in the storage of cholesterol.

The inability to store cholesterol or cholesterol esters in the liver in biotin deficiency may thus be contrasted with the "biotin fatty liver." Some discrepancies in findings require further studies.

J. BIOTIN AND SEX HORMONES

All manifestations of biotin deficiency develop earlier and at a lesser degree of biotin undersaturation in male than in female rats.[1, 4, 8, 64, 66, 67] These differences are obscured in excessively severe depletion of biotin.[64] Gonadectomy produced only slight changes in deficiency symptoms in males. In females it resulted in increased food intake and marked accentuation in severity of symptoms. Removal of the gonads, however, did not entirely eliminate the differences between males and females (adrenals?). Implantation of diethylstilbestrol attenuated, and that of testosterone pellets hastened, the development and increased the severity of biotin deficiency symptoms.[64]

Hertz and Sebrell demonstrated the presence of avidin in the secretion from the mucosal lining of the oviduct in hen and frog. It has been further shown that avidin is present only in the albumen-secreting portion of the oviduct, and its production depends upon ovarian function. No avidin was found in the oviduct of the non-laying hen.[68] Mucosal scrapings from the oviducts of pig, cow, and guinea pig contained no avidin.[69] The secretion of avidin may be induced in the oviduct of the non-laying hen by combined treatment with stilbestrol and either progesterone, desoxycorticosterone acetate, or testosterone propionate.[70, 71]

The fat-soluble biotin-like fraction of the plasma (FSF) (see pp. 565, 566) is increased in the actively laying hen.[72] Total biotin activity, composed of that of true biotin and of the fat-soluble fraction, is elevated fivefold in the blood of the estrogen-treated sexually immature chick. In these chicks the weight of the oviduct is considerably increased but the secretion of avidin is not stimulated. Combined administration of stilbestrol and

[66] P. György, *J. Biol. Chem.* **131**, 733 (1939).
[67] F. Stenquist, *Deutsch. Med. Wochschr.* **54**, 1920 (1928).
[68] R. M. Fraps, R. Hertz, and W. H. Sebrell, *Proc. Soc. Exptl. Biol. Med.* **52**, 140 (1943).
[69] R. Hertz, *Physiol. Rev.*, **26**, 479 (1946).
[70] R. Hertz, R. M. Fraps, and W. H. Sebrell, *Proc. Soc. Expil. Biol. Med.* **52**, 142 (1943).
[71] R. Hertz, R. M. Fraps, and W. H. Sebrell, *Science* **100**, 35 (1944).
[72] W. Trager, *J. Biol. Chem.* **176**, 133 (1948).

progesterone is followed by substantial inhibition of the growth of the genital tract, without any depression in plasma biotin activity. Simultaneously, avidin production becomes apparent in chicks treated with stilbestrol and progesterone (see Table XII). "The role of such metabolites as biotin and avidin in the mechanism of hormone induced tissue-growth remains a challenging enigma.[73]

TABLE XII[73]

EFFECT OF STILBESTROL AND STILBESTROL PLUS PROGESTERONE ON BIOTIN LEVELS IN CHICK PLASMA

	Untreated, 12 samples		Stilbestrol,[a] 13 samples		Stilbestrol plus progesterone,[b] 8 samples	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
Biotin activity[c] (FSF)	3.8	0.79	15	4.4	11	1.8
True biotin[c] (aqueous fraction)	1.3	0.22	8.3	1.8	10	1.3
Oviduct weights	29.0	6.6	1091.0	192.0	380	93.0
Avidin[d]	—		—		+	

[a] Injected subcutaneously with 0.25 mg. of stilbestrol in 0.2 ml. of corn oil daily for 8 days.

[b] Injected subcutaneously with 0.25 mg. of stilbestrol in 0.2 ml. of corn oil and 1.0 mg. of progesterone in 0.2 ml. of corn oil daily for 8 days.

[c] Values expressed as millimicrograms of biotin per milliliter of undiluted plasma (in microbiological tests on *L. casei*).

[d] (−) signifies less than and (+) more than, 0.1 unit of avidin per gram of oviduct (wet weight). A unit of avidin is the amount required to inhibit completely the yeast growth supported by 1 γ of biotin.

K. BIOTIN AND CANCER

Rapidly growing tissues, such as embryonic tissue and cancer, are in general low in biotin. In careful studies West and Woglom[74, 75] compared the biotin content in various tissues and organs, obtained from adult animals, from embryos, and tumors. Their findings are summarized in Table XIII.

In most cases studied, the tumor, as well as the embryonic tissues, contained significantly less biotin than the corresponding tissues in adult animals. In a few cases, such as rabbit skin and connective tissue, the biotin content in adult animals was higher than in the embryo or in tumor tissue originating from the skin or connective tissue. Rat hepatoma is also low in biotin, compared with the high biotin content of normal liver tissue.[76]

[73] R. Hertz, F. G. Dhyse, and W. W. Tullner, *Endocrinology* **45**, 451 (1949).

[74] P. M. West and W. H. Woglom, *Science* **93**, 525 (1941).

[75] P. M. West and W. H. Woglom, *Cancer Research* **2**, 324 (1942).

[76] M. A. Pollack, A. Taylor, A. Woods, R. C. Thompson, and R. J. Williams, *Cancer Research* **2**, 748 (1942).

The biotin content of fetal tissues increases rapidly during the last stage in pregnancy. In rats the adjustment to adult levels takes place during the last 2 to 3 days of pregnancy. The concentration of biotin in the fetuses, which in rats was found to be 0.02 γ per gram of fresh tissue on the thirteenth day of pregnancy, increased fivefold prior to parturition.[77]

The low biotin content in immature tissues, such as fetal or tumor tissue, aroused the interest of several workers as to the possible relation of biotin to tumor growth as such. Du Vigneaud and his associates[78] studied the effect of biotin on the production of primary hepatoma in rats fed 1,1-N-dimethylaminoazobenzene (butter yellow). It had previously been shown that various dietary factors, such as riboflavin and high protein intake, may exert a protective effect on the liver in rats receiving the carcinogen butter yellow. Du Vigneaud and his associates found that the addition of pure biotin to such highly protective rations caused a higher incidence in tumor formation. Thus biotin, under the experimental conditions chosen, seemed to exert a procarcinogenic effect. It was then suggested that biotin deficiency induced by avidin, the egg white injury factor, might be of some therapeutic value on cancer.

TABLE XIII

Comparative Values on the Biotin Content[a] of Adult, Embryo, and Tumor Tissues According to West and Woglom[75]

	Liver			Lung			Skin			Connective tissue		
	Adult	Embryo	Tumor	Adult	Embryo	Tumor	Adult	Embryo	Tumor	Adult	Embryo	Tumor
Rat	4480	1280	2030	—	—	—				2460	1280	544
Mouse	3613	2909	1170	2280	1330	1177				1280	312	556
Rabbit	—	—	—	—	—	—	89	531	360[b]			
									369[c]	134	1330	813

[a] Biotin values given in milligrams per gram of dry tissue.
[b] Rabbit papillomas.
[c] Rabbit skin carcinomas.

In later studies, this assumption was not borne out by animal experiments or by observations on human patients.

Even the original findings on the effect of biotin in rats fed butter yellow could not be duplicated.[79, 80] Admittedly, egg white appeared to afford some protection against hepatoma formation due to butter yellow. Inas-

[77] H. Lewis and G. Everson, *J. Nutrition* **46**, 27 (1952).
[78] V. du Vigneaud, J. M. Spangler, D. Burk, C. J. Kensler, K. Suguira, and C. P. Rhoads, *Science* **95**, 174 (1942).
[79] P. György, Unpublished observations.
[80] B. E. Kline, J. A. Miller, and H. P. Rusch, *Cancer Research* **5**, 644 (1945).

much as this effect was exerted by unheated as well as by heated (avidin-free) egg white, it must be independent of any relation between biotin and avidin.

Furthermore, in mice with spontaneous mammary carcinoma, a diet rich in egg white and avidin was without any beneficial effect. Similar negative results were obtained with a ration containing high levels of egg white in mice with Flexner-Jobling sarcoma or mouse sarcoma 180.[81] In other experiments[75] mice were fed an avidin-containing diet until they developed a severe biotin deficiency with a simultaneous reduction of the biotin levels in the tissues 80 to 90% below normal. Fragments of sarcoma 37 or 180 were introduced subcutaneously into these biotin-deficient mice as well as into normal control mice. All the tumors in both groups of animals grew well and were large and healthy, indicating that severe biotin deficiency was without any effect on the propagation of the tumor tissue.

Attempts were also made to influence cancerous growth in man by a diet containing excessive amounts of egg white or avidin. The results were disappointingly negative.[82, 83]

L. BIOTIN AND RESISTANCE TO DISEASE

Trager[84, 85] has shown that biotin deficiency causes both a prolongation and an increased severity of infection with *Plasmodium lophurae* in chickens and with the same avian malaria plasmodium or with *P. cathemerium* in ducks. A diet deficient in pantothenic acid had no effect on the malaria infections in chickens. Caldwell and György have reported a significant prolongation of infection with *Trypanosoma lewisi* in the albino rat as a result of biotin deficiency.[86] Seeler *et al.*[87] confirmed Trager's findings on biotin deficiency and avian malaria. On the other hand, Seeler and Ott[88] observed that riboflavin deficiency decreased the severity of infection with *P. lophurae* in the chick although the infection was not shortened. Biotin deficiency also decreased the resistance of mice to *Salmonella typhi-murium* infection[89] but had no effect on the susceptibility of Swiss mice to experimental poliomyelitis.[90]

[81] C. J. Kensler, C. Wadsworth, K. Suguira, C. P. Rhoads, K. Dittmer, and V. du Vigneaud, *Cancer Research* **3**, 823 (1943).
[82] C. P. Rhoads and J. C. Abels, *J. Am. Med. Assoc.* **121**, 1261 (1943).
[83] I. I. Kaplan, *Am. J. Med. Sci.* **207**, 733 (1944).
[84] W. Trager, *J. Exptl. Med.* **77**, 557 (1943).
[85] W. Trager, *J. Exptl. Med.* **85**, 663 (1947).
[86] F. E. Caldwell and P. György, *Proc. Soc. Exptl. Biol. Med.* **53**, 116 (1943).
[87] A. O. Seeler, W. H. Ott, and M. E. Gundel, *Proc. Soc. Exptl. Biol. Med.* **55**, 107 (1944).
[88] A. O. Seeler and W. H. Ott, *J. Infectious Diseases* **75**, 175 (1944).
[89] I. J. Klugler, K. Guggenheim, and H. Herrnheiser, *J. Infectious Diseases* **78**, 60 (1946).

During infection of chicks and ducks with avian malaria, significant changes occurred in the concentration in the plasma of free biotin and of the bound, fat-soluble fraction (see p. 566) with biotin-like activity.[84, 85] The concentration of free biotin rose to a peak at about the time of the peak parasite number and then returned to normal in the surviving birds. It attained the highest levels in birds which succumbed to the infection. In contrast, the bound fat-soluble fraction first rose and then fell before death to very low levels, reaching zero in several animals. Biotin as such had no effect on *P. lophurae in vitro*, while plasma protein fractions rela-

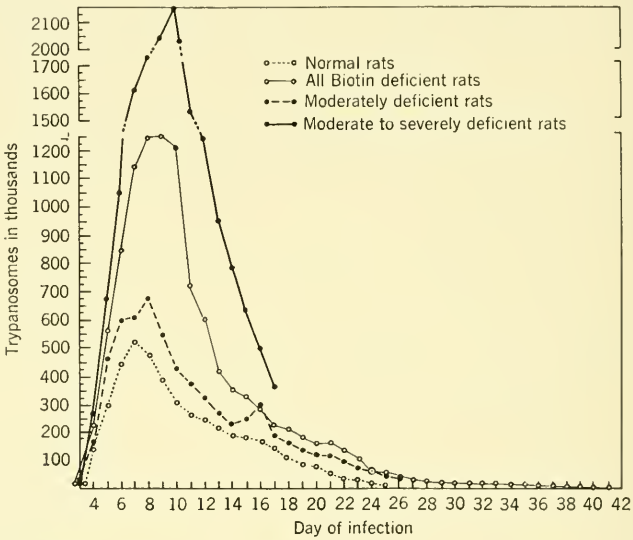

Fig. 9. Mean infections with *Trypanosoma lewisi* in biotin-deficient and norma rats.

tively rich in fat-soluble fraction inhibited multiplication of the malaria parasite.[85] These observations leave the mechanism of decreased resistance to infection in biotin deficiency unexplained.

Caldwell and György[91] observed that individual and mean infections of *Trypanosoma lewisi* within groups of biotin-deficient rats showed longer periods in which the trypanosomes were increasing in number (Fig. 9). This finding, together with delays in the initial trypanolytic crises and delays in terminating the infections, by comparison with the infections in the normal rats, may be best attributed to a delayed or quantitatively or qualitatively deficient production of specific antibodies. Indications were also found that

[90] H. C. Lichtstein, H. A. Waisman, K. B. McCall, C. A. Elvehjem, and P. F. Clark, *Proc. Soc. Exptl. Biol. Med.* **60**, 279 (1945).

[91] F. E. Caldwell and P. György, *J. Infectious Diseases* **81**, 197 (1947).

in rats suffering from terminal stages of biotin deficiency the complement titer of the plasma may be considerably reduced. Rats fed the biotin-deficient diet but given adequate biotin daily from the time of weaning had infections with *T. lewisi* that were no different from the infections in rats fed a normal stock diet. Excess biotin administered to normal rats had no protective or curative effects on infections with *T. lewisi* or *T. equiperdum*.[91]

XI. Pathology

PAUL GYÖRGY

The visible, gross pathological changes in biotin deficiency in various animals and man have been described as scaly, seborrheic dermatoses, often accompanied by alopecia.

The microscopic picture was best studied in rats.[1-4] The skin shows extensive hyperkeratosis, some parakeratosis, acanthosis, and edema. Broken hair shafts and serous crusts are intermingled with the hyperakeratotic lamellae. There is dilatation of the follicular shafts, and the glandular orifices are often plugged with hyperkeratotic material. Surprisingly little cellular infiltration accompanies the marked changes in the epidermis and the edema in the corium. The connective tissue is slightly edematous and dissociated. Most striking is the presence of numerous dilated and hyperemic blood vessels throughout the corium. There is no evidence of endovasculitis and no perivascular infiltration. In the late stage of the disease there is some atrophy of the epidermis, which contrasts with the abundant hyperkeratosis. There is an excessive amount of sudanophilic fat in the hyperkeratotic lamellae; as the skin lesions undergo involution the normal epithelium is restored with slight damage to the fibrous connective tissue but with no disturbance of the elastic fibers.

In contrast to the picture seen in rats, keratosis is only moderate in the mouse suffering from biotin deficiency. The pilosebaceous orifices are dilated in deficient rats but only rarely in the mouse. The stratum corneum is sudanophilic in deficient rats, but it is only mildly so in the mouse. The subcutaneous fat depots are resorbed in deficient rats, but they remain virtually unchanged in the mouse. In the sebaceous cells, the stored lipid droplets are much larger than in those of normal animals. There is abundant

[1] M. Sullivan and J. Nicholls, *Arch. Dermatol. and Syphilol* **45**, 295 (1942).

[2] C. M. Findlay and R. O. Stern, *Arch. Disease Childhood* **4**, 1 (1929).

[3] P. György, M. Sullivan, and H. T. Karsner, *Proc. Soc. Exptl. Biol. Med.* **37**, 313 (1937).

[4] M. Sullivan, L. Kolb, and J. Nicholls, *Bull. Johns Hopkins Hosp.* **70**, 177 (1942).

cellular fragmentation at the periphery of sebaceous glands, and the lipids are apparently phagocytized by histiocytes. Lipids may also be found in melanin-bearing cells around the sebaceous cells.[5]

No specific changes were found in the nervous system or in the muscles in biotin-deficient rats. The muscles may be atrophic but without signs of true degeneration.[6]

XII. Pharmacology

PAUL GYÖRGY

Administration of biotin either in a single massive dose or in multiple large doses produces essentially no striking pharmacodynamic action.[1, 2]

Large single doses of *dl*-biotin (1 g. per kilogram) were without any detectable toxic effect in mice. Multiple large doses given intravenously to dogs or *per os* to mice and normal rats produced no signs of toxicity.

Large doses of biotin had no effect on the blood pressure, heart rate, or respiration in anesthetized cats or in rats. Relatively high concentrations of biotin produced no effect on strips of guinea pig, rat, or rabbit uterus, on rabbit or rat intestine, or on frog's heart when perfused *in situ*.

No signs of local irritation were observed after application of biotin to the cornea (rabbit) or after intradermal (guinea pigs) or intramuscular (rabbit) injections.

The secretion of free hydrochloric acid by the gastric glands appeared to be depressed by *d*-biotin. Renal function or metabolic rate remained unchanged after administration of a large single oral dose of *d*-biotin. Repeated oral doses of biotin produced no changes in hepatic or renal function, blood fibrinogen, rectal temperature, sensitivity of nerve endings, skin regeneration, or healing of scalded skin in the normal rat.

Pharmacological studies on biotin-deficient rats revealed a few interesting facts.[3] The amplitude of contractions of the isolated uterus or intestine of the biotin-deficient rat is less than that of the normal animal of the same age. Biotin added to the Ringer-Locke solution in which the isolated organs were suspended did not alter the contractions.

The intravenous administration of large doses of biotin to the anesthetized biotin-deficient animal has no effect on heart rate, blood pressure,

[5] W. Montagna, *Proc. Soc. Exptl. Biol. Med.* **73**, 127 (1950).
[6] J. H. Shaw and P. H. Phillips, *Proc. Soc. Exptl. Biol. Med.* **51**, 406 (1942).
[1] J. L. Schmidt and M. Landy, *Proc. Soc. Exptl. Biol. Med.* **49**, 82 (1942).
[2] P. J. Crittenden, *Arch. Intern. Pharmacodynamie* **76**, 417 (1948).
[3] P. J. Crittenden, *Arch. Intern. Pharmacodynamie* **77**, 29 (1948).

intestinal circulation, respiration, or secretion of gastric acid. A single oral dose of biotin to the biotin-deficient rat has no influence on metabolic rate or renal function. Repeated oral doses appear to lower the metabolic rate A slight delay in the healing time of injured skin areas was apparent after local or oral application of biotin to biotin-deficient rats.

XIII. Requirements

PAUL GYÖRGY

Biotin is one of the most powerful catalysts of several metabolic reactions, needed only in minute amounts. As such it is apparently required by animals, plants, and by a large number of bacteria. In animals living in symbiosis with some biotin-producing bacteria, it becomes difficult to assess the exogenous requirements.

In bacteria, the synthesis of aspartic acid and that of oleic acid is intimately linked with the presence of biotin. Several other metabolites, the production of which may be catalyzed by biotin, are either apparently dispensable or else can be obtained through alternate pathways.

A. YEASTS AND OTHER FUNGI

Biotin is essential for the growth of many strains of *Saccharomyces cerevisiae*[1, 2] and for the majority of a large number of other yeasts tested.[3-6] Some of the analogs of biotin, such as desthiobiotin (see p. 562), may replace biotin for many yeasts but not for bacteria.[7] Biotin definitely increases the production of alcohol by yeast.[8] The uptake of biotin is correlated with the requirement in different species of yeasts. Some strains of yeasts, such as *Torulopsis utilis* and *Hansenula anomala*, are able to synthesize biotin and do not utilize the biotin of the medium.[9]

Biotin was found to be essential for a number of molds, including *Lophiodermum pinastri* and *Ashbya* (*Nematospora*) *gossypii*,[10] *Trichophyton album*

[1] R. J. Williams, R. E. Eakin, and E. E. Snell, *J. Am. Chem. Soc.* **62**, 1204 (1940).
[2] L. H. Leonian and V. G. Lilly, *Am. J. Botany* **29**, 459 (1942).
[3] P. R. Burkholder, *Am. J. Botany* **30**, 206 (1943).
[4] P. R. Burkholder and D. Moyer, *Bull. Torrey Botan. Club* **70**, 372 (1943).
[5] P. R. Burkholder, I. McVeigh, and D. Moyer, *J. Bacteriol.* **48**, 385 (1944).
[6] A. S. Schultz and L. Atkin, *Arch. Biochem.* **14**, 369 (1947).
[7] J. L. Stokes and M. Gunness, *J. Biol. Chem.* **157**, 121 (1945).
[8] V. de Souza and M. Sreenivasaya, *J. Sci. Med. Research* (*India*) **4**, 384 (1945).
[9] Wei-Shen Chang and W. H. Peterson, *J. Bacteriol.* **58**, 33 (1949).
[10] F. Kögl and N. Fries, *Hoppe-Seyler's Z. physiol. Chem.* **249**, 93 (1937).

and *Hypholoma fasciculare*,[11, 12] *Cremothecium ashbyii*,[13] *Neurospora sitophila* and its "pyridoxineless" mutant,[14] *Marasmius androsaceus*,[15] *Ascoidea rubescens*, *Ophiostoma fagi*, *O. piliferum*, and *Mitirula paludosa*.[16] The growth of *Penicillium digitatum* is stimulated by biotin, more so above pH 6.5 than at pH 3.0.[17] Some fungi may synthesize biotin. For instance, *Phycomyces blakesleanus* on a synthetic medium containing asparagine and glucose produced considerable amounts of biotin which diffused and accumulated in the medium.[18] *Pencillium chrysogenum* may also synthesize biotin, but only in small amounts.[16]

B. BACTERIA

Biotin was found to be essential for the growth of *Lactobacillus helveticus*,[19] *L. arabinosus*[20] and all species of *Lactobacilli* tested.[21] As previously shown (p. 562) *Lactobacilli* may no utilize desthiobiotin, and their use of other homologs shows various differences. Biotin methyl ester is required in relatively higher concentration by *L. helveticus* than is free biotin. The requirement of three strains of *Leuconostoc*, *L. mesenteroides*, *L. dextranicum*, *L. dextranicum elai*, for biotin depends on the carbohydrate in the medium. They will grow in the absence of biotin on a sucrose medium, but not when sucrose is replaced by invert sugar, glucose, or fructose.[22] Biotin was found to be essential for the following bacteria: *B. radicicola*, *Rhizobium trifolii*[23, 24] *Staphylococcus aureus*,[25] *Streptobacterium plantarum*,[26] and several species of *Propionibacteria*.[27] It is required for optimal growth by a large number of strains of *Clostridia*,[28, 28a, b] including *Cl. kluyveri*[29] and *Cl. aceto-*

[11] W. H. Schopfer and S. Blumer, *Compt. rend. soc. phys. et hist. nat.* (*Genève*) **59**, 106 (1942).
[12] W. H. Schopfer and S. Blumer, *Ber. schweiz. botan. Ges.* **53**, 409 (1943).
[13] W. H. Schopfer, *Helv. Chim. Acta* **27**, 1017 (1944).
[14] J. L. Stokes, J. W. Foster, and C. R. Woodward, *Arch. Biochem.* **2**, 235 (1943).
[15] G. Lindeberg, *Symbolae Botan. Upsalienses*, **8**, No. **2**, 1 (1944).
[16] F. W. Tanner, S. E. Pfeiffer, and J. M. van Lanen, *Arch. Biochem.* **8**, 29 (1945).
[17] R. C. Wooster and V. H. Cheldelin, *Arch. Biochem.* **8**, 311 (1945).
[18] W. H. Schopfer, *Z. Vitaminforsch.* **14**, 42 (1944).
[19] G. M. Shull, B. L. Hutchings, and W. H. Peterson, *J. Biol. Chem.* **142**, 913 (1942).
[20] L. D. Wright and H. R. Skeggs, *Proc. Soc. Exptl. Biol. Med.* **56**, 95 (1944).
[21] M. Rogosa, R. P. Tittsler, and D. S. Geib, *J. Bacteriol.* **54**, 13 (1947).
[22] W. W. Carlson and V. Whiteside-Carlson, *Proc. Soc. Exptl. Biol. Med.* **71**, 416 (1949).
[23] R. Nilsson, G. Bjalfoe, and D. Burström, *Naturwissenschaften* **27**, 389 (1939).
[24] P. M. West and P. W. Wilson, *Science* **89**, 607 (1939).
[25] F. Kögl and W. J. van Wagtendonk, *Rec. trav. chim.* **57**, 747 (1938).
[26] R. Kuhn and K. Schwarz, *Ber.* **74**, 1617 (1941).
[27] R. C. Thompson, *J. Bacteriol.* **46**, 99 (1943).
[28] J. O. Lampen and W. H. Peterson, *Arch. Biochem.* **2**, 443 (1943).
[28a] R. E. Feeney, J. H. Mueller, and P. A. Miller, *J. Bacteriol.* **46**, 563 (1943).

butylicum.[30, 31] *B. macerans* and *B. aectoethylicus* require thiamine and biotin, *B. polymyxa* only biotin of the vitamin B complex.[32] For *Neisseria sicca* biotin is apparently the only growth factor required.[33]

The essential character of biotin for a given bacterial species may be studied by the effect of avidin added to the medium in an amount sufficient to bind all available biotin. No growth will indicate that biotin is an essential nutrient for the microorganism in question. Several homologs, precursors, or in particular oleic acid and related acids (see p. 564) will support growth for a large number of bacteria, even in the absence of biotin, or at least they will materially fill the requirement for biotin.

A number of bacteria are able to synthesize biotin. The amount of biotin found in the five bacteria, *Aerobacter aerogenes, Serratia marcescens, Pseudomonas fluorescens, Proteus vulgaris*, and *Clostridium butylicum*, was calculated to range from 420 to 1800 molecules per cell.[34]

C. INSECTS

Optimal growth was maintained only in the presence of biotin in *Tribolium confusum*,[35-38] *Sitodrepa panicea, Lasioderma serricorne*, and *Ptinus tectus*.[39] Biotin is apparently not required by *Silvanus surinamensis*.[39] Tribolium can utilize not only biotin but the diaminocarboxylic acid prepared from biotin. The sterilized larvae of *Lasioderma* have shown less satisfactory growth in the absence of biotin than have normal larvae. Thus, in the absence of symbionts biotin may become a more important nutrient.[40] The mosquito larvae (*Aëdes aegypti*) require biotin for their growth. It may, however, be replaced by oleic acid or lecithin.[41, 42]

The addition of raw egg white or avidin to the diet of the larvae of *Corcyra cephaloneca* (rice moth) inhibited growth and caused death of the larvae in 28 days. Biotin added to the diet, even 14 days after feeding with

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[28b] C. Lamanna and C. Lewis, *J. Bacteriol.* **51**, 398 (1946).
[29] B. T. Bornstein and H. A. Barker, *J. Bacteriol.* **55**, 222 (1948).
[30] R. Reyes-Teodoro and M. N. Michelson, *Arch. Biochem.* **4**, 291 (1944).
[31] R. Reyes-Teodoro and M. N. Michelson, *Arch. Biochem.* **6**, 471 (1945).
[32] H. Katznelson, *J. Bacteriol.* **48**, 495 (1944).
[33] Z. J. Ordal and R. K. Busch, *J. Bacteriol.* **51**, 791 (1946).
[34] H. McIlwain, *Nature* **158**, 898 (1946).
[35] G. Fraenkel and M. Blewett, *Nature* **149**, 301 (1942).
[36] G. Fraenkel and M. Blewett, *Nature* **150**, 177 (1942).
[37] G. Fraenkel and M. Blewett, *Nature* **151**, 703 (1943).
[38] H. Rosenthal and T. Reichstein, *Nature* **150**, 546 (1942).
[39] G. Fraenkel and M. Blewett, *Biochem. J.* **37**, 686 (1943).
[40] M. Blewett and G. Fraenkel, *Proc. Roy. Soc. (London)* **B132**, 212 (1944).
[41] W. Trager, *J. Biol. Chem.* **176**, 1211 (1949).
[42] L. Golberg, B. de Meillon, and M. Lavoipierre, *J. Exptl. Biol.* **21**, 90 (1945).

egg white or avidin started, will bring about resumption of normal growth in the larvae.[43]

D. HIGHER ANIMALS AND MAN

For reasons previously mentioned it is very difficult and at present barely possible to give a correct estimate for the biotin requirement in animals and man. In the relevant literature the figures given represent in general the recommended minimal daily dose, with wide variations.

Man	150 to 300 γ per day[44]
Rat	0.5 to 3 γ per day[45, 46]
Mouse	8 γ per 100 g. of diet[47]
Chick	0.65 to 1 γ per day[48-50] or
	7 to 10 γ per 100 g. of diet[51]
Turkey	40 "rat units"[52]
Pig	100 γ per day[53]
Monkey	20 γ per day[48]

There is a fairly definite but not completely satisfactory correlation[54] between body weight and daily biotin requirement (per kilogram of body weight).

Even if the quantitative daily biotin requirement could be exactly determined and defined for a given case, the results obtained would apply only for the condition existing at the time of the observation. Requirements for essential nutrients, and therefore also for biotin, are not rigidly fixed quantities. They vary according to the internal and external environment of man and animals at a given moment. Thus the above figures should be considered as approximations, subject to wide changes and fluctuations.

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